

**EXAMINING THE METHODS OF PLANT WATER EXTRACTION AND STABLE  
ISOTOPES ANALYSIS FOR ECOHYDROLOGICAL STUDIES.**

A Thesis Submitted to the College of Graduate and Postdoctoral Studies

In Partial Fulfillment of the Requirements

For the Degree of Master of Environment and Sustainability

In the School of Environment and Sustainability

University of Saskatchewan

Saskatoon, Canada.

By

Cody Millar

© Copyright Cody Millar, May 2018. All rights reserved.

## **PERMISSION TO USE STATEMENT**

In presenting this thesis in partial fulfillment of the requirements for a Postgraduate degree from the University of Saskatchewan, I agree that the Libraries of this University may make it freely available for inspection. I further agree that permission for copying of this thesis in any manner, in whole or in part, for scholarly purposes may be granted by the professor who supervised my thesis work or, in their absence, by the Head of the Department or the Dean of the College in which my thesis work was done. It is understood that any copying or publication or use of this thesis or parts thereof for financial gain shall not be allowed without my written permission. It is also understood that due recognition shall be given to me and to the University of Saskatchewan in any scholarly use which may be made of any material in my thesis.

Requests for permission to copy or to make other uses of materials in this thesis/dissertation in whole or part should be addressed to:

Executive Director

School of Environment and Sustainability

University of Saskatchewan

Room 323, Kirk Hall

117 Science Place

Saskatoon, Saskatchewan S7N 5C8 Canada

Or

Dean

College of Graduate and Postdoctoral Studies

University of Saskatchewan

116 Thorvaldson Building, 110 Science Place

Saskatoon, Saskatchewan S7N 5C9

Canada

## **DISCLAIMER**

The software R was used to meet the thesis requirements for the degree of Master of Environment and Sustainability at the University of Saskatchewan. Reference in this thesis/dissertation to any specific commercial products, process, or service by trade name, trademark, manufacturer, or otherwise, does not constitute or imply its endorsement, recommendation, or favoring by the University of Saskatchewan. The views and opinions of the author expressed herein do not state or reflect those of the University of Saskatchewan and shall not be used for advertising or product endorsement purposes.

## ABSTRACT

The stable isotope ratios of hydrogen and oxygen ( $^2\text{H}/^1\text{H}$  ( $\delta^2\text{H}$ ) and  $^{18}\text{O}/^{16}\text{O}$  ( $\delta^{18}\text{O}$ )) are an important, and widely applied set of tracers, used to trace water movement through the soil-plant-atmosphere continuum. They are employed in a variety of physiological, ecohydrological, and biogeochemical studies. An accurate understanding of the global water balance will be critical in a world undergoing rapidly changing climatic conditions. Specifically, understanding how, where, and when plants get their water from the soil is crucial to our management of water resources, especially considering a future where the availability of fresh water for irrigation may be limited. For studies investigating plant water use via stable isotopes, water (analyte) extraction and analysis are a critical step. Several methods exist to extract and analyze plant and soil analytes for stable isotope composition. However, new work has called into question the validity of using these methods interchangeably when extracting soil bound analytes. Within this thesis work, I began by inter-comparing six methods of plant analyte extraction for stable isotope analysis of  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  values, to determine if these extraction systems return accurate, interchangeable results. I then examined how the presence of co-extracted organic contaminants like methanol and ethanol effected stable isotope results returned by the Los Gatos Research off axis-integrated cavity output spectroscopy (OA-ICOS) system. Additionally, I assessed the effectiveness of the Absorption plot and the Spectra Fit Residuals plot, sub components of the OA-ICOS system, at detecting organic contamination during vapour-mode analysis.

Key findings of this work are: (1) The tested methods of plant analyte extraction returned significantly different ( $p \leq 0.05$ ) results; (2) Direct vapour equilibration may be a useful approach for plant analyte extraction-analysis due to potential limited interference from organic compounds, rapid sample throughput and field portability; (3) I confirm that methanol and ethanol drastically impact returned stable isotope results during analysis by spectrometric methods (OA-ICOS), but contrary to the findings of previous work, methanol caused OA-ICOS results to skew positively, while ethanol caused OA-ICOS result to skew negatively, relative to the control water isotopic composition; and (4) the Spectra Fit Residuals plot is a useful tool for the detection of organic contamination during vapour-mode analysis. These findings will have direct implications for studies utilizing stable isotope data generated from plant extracted water.

## PERMISSION TO REPRODUCE

Permission to reproduce a published manuscript in this dissertation was acquired for the following publication:

1. Millar C, Pratt D, Schneider D J, McDonnell J J. A comparison of extraction systems for plant water stable isotope analysis. *Rapid Commun Mass Sp* 2018; 32(13):1031-1044.

Publisher: Rapid Communications in Mass Spectrometry (Wiley)

Copyright holder permission obtained from: Rapid Communications in Mass Spectrometry (Wiley)

## **DEDICATION**

This thesis is dedicated to my partner Charlotte Dykes, my parents Brian and Isabel Millar, my brother Riley Millar, and to my grandparents Corrine and Donald Leask & Alice and John Millar.

## ACKNOWLEDGEMENTS

My supervisor Dr. Jeffrey J. McDonnell, for his belief in me, his generous support, and his guiding wisdom, helping me to produce high quality work as a master's candidate. My co-authors Dr. Dyan Pratt (Global Institute for Water Security-University of Saskatchewan) and Dr.

Dave Schneider (Global Institute for Food Security-University of Saskatchewan) for their technical support and theoretical insights. Dr. Natalie Orlowski for the inspiration to begin this work. Kim Janzen for her tireless efforts providing isotopic analysis and support in the lab. The Global Institute for Water Security, the School of Environment and Sustainability (SENS), and the Natural Sciences and Engineering Research Council of Canada-CREATE program for financial support during my Masters. My colleagues in the McDonnell watershed hydrology lab, and at the University of Georgia Athens: Marcel Gaj, Magali Nehemy, Chris Gabrielli, Anna Coles, Veva McDonnell, Lauren Gill, and Seth Younger for their valuable input and stimulating intellectual discourse. My committee members Dr. David J Schneider, Dr. Helen Baulch, Dr.

Bingchen Si, and Dr. Geoff Koehler for their guidance throughout the development and culmination of my master's research. My parents Brian and Isabel for their encouragement and support throughout my undergraduate and graduate work.

Finally, and most importantly, to my partner Charlotte Dykes for her love and incredible support these past two years.

Thank you all!

## TABLE OF CONTENTS

PERMISSION TO USE STATEMENT.....	i
DISCLAIMER.....	ii
ABSTRACT.....	iii
PERMISSION TO REPRODUCE.....	iv
DEDICATION.....	v
ACKNOWLEDGEMENTS.....	vi
TABLE OF CONTENTS .....	vii
LIST OF TABLES.....	xi
LIST OF FIGURES .....	xii
LIST OF ABBREVIATIONS .....	xiii
Chapter 1: Introduction.....	1
1.0 Introduction.....	1
1.1 The Use of Stable Isotopes Found in Water as a Conservative Tracer.....	2
1.2 Plant Analyte Extraction Methods.....	4
1.3 Extraction Method Comparisons.....	5
1.4 Plant Analyte Extraction and Stable Isotope Analysis Problems.....	7
1.5 Ecohydrological Separation in Plant Water Use.....	8
1.6 Research Gap.....	10
1.7 Objectives and Hypotheses.....	11
1.8 References.....	14
Chapter 2: A Comparison of Extraction Systems for Plant Water Stable Isotope Analysis.....	20
2.1 Abstract.....	20



2.2 Introduction.....	21
2.3 Materials and Methods.....	22
2.3.1 <i>Plant Water Extraction Techniques and Extraction Parameters</i> .....	23
2.3.2 <i>Isotope Analyses</i> .....	27
2.3.3 <i>Quantifying Methanol and Ethanol Content</i> .....	28
2.3.4 <i>Statistical Analyses</i> .....	29
2.4 Results.....	30
2.4.1 <i>Plant Portion and Extraction Method Differences</i> .....	31
2.4.2 <i>Co-extracted Organics and Resultant Differences Between OA-ICOS and IRMS Analyses</i> .....	31
2.4.3 <i>Variance Analysis</i> .....	33
2.4.4 <i>Extraction Efficiencies and Sample Throughput</i> .....	34
2.5 Discussion.....	35
2.5.1 <i>Methodological Controls on Accessible Water Pools within the Plant</i> .....	35
2.5.2 <i>On the Accuracy of Extraction Methods Based on Co-extracted Organic Compound Content</i> .....	36
2.5.3 <i>Extraction Methods and their Effects on <math>\delta^{18}\text{O}</math> and <math>\delta^2\text{H}</math> Values</i> .....	38
2.5.4 <i>Extraction Method Intercomparison</i> .....	40
2.5.5 <i>Limitations of our Study Approach and Recommendations</i> .....	41
2.6 Conclusions.....	42
2.7 Acknowledgments.....	43
2.8 Transition Statement.....	44
2.9 Author Contributions.....	44

2.10 References.....	44
2.11 Tables and Figures.....	48
Chapter 3: Further Experiments Comparing Direct Vapour Equilibration and Cryogenic Vacuum Distillation for Plant Water Stable Isotope Analysis.....	57
3.1 Letter to the Editor.....	57
3.2 Acknowledgements.....	64
3.3 Transition Statement.....	65
3.4 Author Contributions.....	65
3.5 References.....	65
3.6 Tables and Figures.....	67
Chapter 4: Differences Between Off-axis Integrated Cavity Output Spectroscopy and Isotope Ratio Mass Spectrometry for Organically Contaminated Samples in Liquid and Vapour Mode.....	71
4.1 Abstract.....	71
4.2 Introduction.....	72
4.3 Materials and Methods.....	74
4.3.1 <i>Isotopic Analyses</i> .....	75
4.3.2 <i>Use of Absorption and Spectra Fit Residuals Plots for Contamination Detection</i> .....	77
4.3.3 <i>Data Analysis</i> .....	77
4.4 Results.....	78
4.4.1 <i>Effects of Contamination on Liquid Water Analysis</i> .....	78

4.4.2 Ability of Absorption and Spectra Fit Residuals Plots to Detect Contaminants.....	81
4.5 Discussion.....	82
4.5.1 Our Findings in the Context of Previous Work.....	82
4.5.2 Absorption and Spectra Fit Residuals Plots as Contaminant Detectors.....	86
4.6 Conclusion.....	87
4.7 Acknowledgments.....	88
4.8 Transition Statement.....	88
4.9 Author Contributions.....	88
4.10 References.....	89
4.11 Tables and Figures.....	91
Chapter 5: Conclusion.....	101
5.1 Findings of Chapter 2.....	102
5.2 Findings of Chapter 3.....	104
5.3 Chapter 2 and 3 Implications and Limitations.....	104
5.4 Findings of Chapter 4.....	105
5.5 Chapter 4 Implications and Limitations.....	106
5.6 Future Recommendations.....	107
5.7 References.....	108

## LIST OF TABLES

<b>Table 2.1.</b> Means and standard deviations of $\delta^2\text{H}$ and $\delta^{18}\text{O}$ ratios from spring wheat Chp2.....	48
<b>Table 2.2.</b> Results of methanol and ethanol content analysis by GC-FID.....	49
<b>Table 2.S1.</b> Compilation of soil characteristics.....	55
<b>Table 2.S2.</b> Irrigation water characteristics.....	56
<b>Table 3.1.</b> Means and standard deviations of $\delta^2\text{H}$ and $\delta^{18}\text{O}$ ratios from spring wheat Chp 3.....	67
<b>Table 4.1.</b> Means and standard deviations $\delta^2\text{H}$ and $\delta^{18}\text{O}$ ratios from control and MeOH-water and EtOH-water contaminated samples.....	91
<b>Table 4.2.</b> Relative error of $\delta^2\text{H}$ and $\delta^{18}\text{O}$ values measured by IRIS and OA-ICOS.....	92

## LIST OF FIGURES

<b>Figure 2.1.</b> Photographic compilation of extracted analyte.....	50
<b>Figure 2.2.</b> Soil pore water stable isotope ratio mean and standard deviations.....	51
<b>Figure 2.3.</b> Dual isotope plot of all results.....	52
<b>Figure 2.4.</b> Dual isotope plots of extracted plant analyte by method of extraction.....	53
<b>Figure 2.5.</b> ANOVA results detailing significant differences.....	54
<b>Figure 3.1.</b> Photographic compilation of spring wheat growth stages for container-1.....	68
<b>Figure 3.2.</b> Dual isotope plots of extracted plant water $\delta^2\text{H}$ and $\delta^{18}\text{O}$ values.....	69
<b>Figure 3.3.</b> Details of variance testing between DVE and CVD-2.....	70
<b>Figure 4.1.</b> Screenshot of ‘Absorption’ plot and ‘Spectra Fit Residual’ plot: no contamination.....	93
<b>Figure 4.2:</b> Dual isotope plots of stable isotope results produced by IRMS for MeOH and EtOH contaminated samples.....	94
<b>Figure 4.3.</b> Dual isotope plots of stable isotope results produced by OA-ICOS for MeOH and EtOH contaminated samples.....	95
<b>Figure 4.4.</b> $\delta^2\text{H}$ errors associated with MeOH and EtOH contamination.....	96
<b>Figure 4.5.</b> $\delta^{18}\text{O}$ errors associated with MeOH and EtOH contamination.....	97
<b>Figure 4.6.</b> MeOH/water and EtOH/water mixture results as analyzed via IRMS and OA-ICOS. ....	98
<b>Figure 4.7.</b> Photographic compilation of OA-ICOS Absorption (Ab) plot and Spectral Fit Residual plot under varying levels of MeOH contamination.....	99
<b>Figure 4.8.</b> Photographic compilation of OA-ICOS Absorption (Ab) plot and Spectral Fit Residual plot under varying levels of EtOH contamination.....	100

## LIST OF ABBREVIATIONS

Ab	absorption plot
ANOVA	analysis of variance
C1	container - 1
C2	container - 2
CVD	cryogenic vacuum distillation
CVD-1	cryogenic vacuum distillation type 1 based on Orłowski <i>et al.</i> 2013
CVD-2	cryogenic vacuum distillation type 2 based on Koeniger <i>et al.</i> 2011
DIw	de-ionized water
DVE	direct vapour equilibration
EtOH	ethanol
EM	electromagnetic
GC-FID	gas chromatograph - flame ionization detection
GMWL	global meteoric water line
HDO	deuterium
HPMS	high pressure mechanical squeezing
ICP	inductively coupled plasma (atomic emission spectroscopy).
IRIS	isotope ratio infrared spectroscopy
IRMS	isotope ratio mass spectroscopy
IWA-45EP	isotope water analyzer - model number
LGR	Los Gatos Research
LMWL	local meteoric water line
LWIA-SCI	liquid water isotope analyzer - spectral contamination identifier
MAB	maximum accepted bias
ME-OA-ICOS	microwave extraction - off axis - integrated cavity output spectroscopy
MeOH	methanol
NSD	not significantly different
OA-ICOS	off axis - integrated cavity output spectroscopy
PP	plant portions
ppmV	parts per million by volume

sd	standard deviation
SD	significantly different
SFR	Spectra Fit residuals Plot
SLAP	standard light Antarctic precipitation
TC/EA-IRMS	thermal conversion/elemental analyzer - isotope ratio mass spectrometry
TWWH	two water worlds hypothesis
VOC	volatile organic compound(s)
VSMOW	Vienna standard mean ocean water
WS-CRDS	wavelength scanned - cavity ring down spectroscopy (aka CRDS)

## Chapter 1: Introduction

### 1.0 Introduction

The stable isotope ratios of hydrogen and oxygen ( $^2\text{H}/^1\text{H}$  ( $\delta^2\text{H}$ ) and  $^{18}\text{O}/^{16}\text{O}$  ( $\delta^{18}\text{O}$ )) are widely applied to a variety of ecohydrological, biogeochemical and climatological studies, typically as conservative tracers.<sup>[1-4]</sup> The stable isotope approach to tracing plant water sources has been widely used since the pioneering work of Dawson and Ehleringer<sup>[5]</sup>. In such studies, precipitation, stream water, mobile soil water, bulk soil water, and plant water are collected and analyzed for their stable isotope composition. Conclusions about plant water sourcing are then drawn from the connections between plant and soil water isotopic compositions. A variety of methods exist that are used to access plant and soil pore water for stable isotope analysis<sup>[3, 4, 6]</sup>. However, the need for a better understanding of the inter-comparability of these methods has been noted<sup>[4, 7]</sup>. In response to this call, recent work has questioned the validity of using these methods for soil water extraction, and subsequent isotope analysis, interchangeably<sup>[3, 8]</sup>. Orlowski *et al.*<sup>[3]</sup> found that returned pore water's isotopic composition is significantly affected by the method of extraction applied.

A formal inter-method comparison of the systems used to extract plant analytes for isotopic analysis of  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  values has not yet been carried out. The findings of Orlowski *et al.*<sup>[3]</sup> imply that this is needed. The term ‘analyte’ is used here to define the liquid that is extracted from plants for stable isotope analysis of oxygen and hydrogen isotopes, because the term ‘water’ is not a truly representative description of this liquid, which contains other substances besides  $\text{H}_2\text{O}$ . The analyte extracted from plants is composed of plant bound water as well as soluble organic compounds, which have their own unique isotopic compositions. Accurate analyses of plant analyte stable isotope compositions are of particular importance in making accurate conclusions about the ecohydrological dynamics of plant water use. Recent work utilizing the stable isotope ratios of hydrogen and oxygen has shown evidence of water uptake dynamics in trees described as the ‘two water worlds’ hypothesis (TWWH)<sup>[7, 9, 10]</sup>. This phenomenon, also known as ecohydrological separation, is defined as “vegetation and streams returning different pools of water to the hydrosphere”<sup>[7]</sup>. The TWWH has important implications



for the management of water resources in forestry and agriculture. For example, if crops are preferentially utilizing the more tightly bound, immobile soil water pool; as in the TWWH, then there is a possibility that the irrigation excess, the mobile soil water pool, is being wasted. Little work has yet been done to explore the presence or absence of ecohydrological separation in crops such as spring wheat (*Triticum aestivum* L.). For future research that seeks to understand water uptake dynamics in economically valuable cereal crops like wheat, it is critical that our investigative tool kits be thoroughly vetted. Accurate interpretation of plant water sources, as well as the isotopic composition of the water they return to the hydrosphere through transpiration are important components of being able to understand and manage the global hydrological cycle and plan for future outcomes based on optimized models.

My thesis falls within a multidisciplinary context and a variety of research themes are interconnected within the problems addressed. In this introduction, I will detail the use of stable isotopes as tracers in environmental studies. Next, I review the various systems used to extract analytes from plants for stable isotope analysis. Further, I detail the results from previous extraction method comparisons, and the problems related to plant analyte extraction and stable isotope analysis. A discussion of the recently discovered phenomenon of ecohydrological separation will follow. Finally, I will address the research gap related to these interdisciplinary topics and show how my thesis work seeks to fill these gaps.

### **1.1 The Use of Stable Isotopes Found in Water as Conservative Tracers**

The stable isotopes of hydrogen and oxygen (within the water molecule) are now widely applied to a variety of ecohydrological, biogeochemical and climatological studies <sup>[1-4]</sup>. Stable isotope approaches have been used to determine water sources and water resource competition of plants <sup>[11-13]</sup>, quantifying plant responses to precipitation changes <sup>[14]</sup>, and partitioning evapotranspiration fluxes <sup>[15-17]</sup>. Studies have also used stable isotopes found in water to derive zones of root activity in soils and root water uptake patterns <sup>[13, 18-21]</sup>. In agriculturally-focused research, stable isotopes have been used to determine the water use efficiency of various crops <sup>[22, 23]</sup>. The use of the stable isotopes of hydrogen and oxygen has become a common approach in

investigations examining root water uptake patterns and water use efficiency of agricultural crops <sup>[16, 21, 24]</sup>.

One such crop, spring wheat; whose estimated global trade value is greater than all other cereal crops combined, is widely irrigated <sup>[25]</sup>. Understanding water uptake patterns in this crop is therefore an important aspect of water resource management. With the rise of global climate change and increasing population, it is difficult to assess the availability of fresh water for future irrigation use <sup>[26]</sup>. By some estimates, a rise in global temperature of 2 °C will result in an increase of 40% for the number of people living under absolute water scarcity, defined as < 500 m<sup>3</sup> per capita per year <sup>[27]</sup>. Shifting climates may result in increased fresh water resources in some regions, while limiting it in others <sup>[28]</sup>. As food security is tightly linked to water availability <sup>[27, 29]</sup> it will be critical to understand crop water use patterns so that producers can be informed of best irrigation practices. The stable isotopes of hydrogen and oxygen have thus far proven to be a powerful tool for understanding root water uptake patterns.

The distribution of a plant's root systems throughout the soil profile constrains a plant's ability to access soil bound water. However, the primary sourcing of water in the soil profile cannot be determined by root size, amount, and distribution alone <sup>[21, 30, 31]</sup>. The range of accessible soil water for plants and crops like wheat, is determined by its final rooting depth. A common method, within the stable isotope approach, to determining a plant's water source is the direct inference method, wherein the plant water isotopic signature ( $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  values) is matched to that of the soil water isotope signal <sup>[21, 32]</sup>. However, in some cases, multiple signals may be present in the soil that match the plant water signal. In such cases, Zhao *et al.* <sup>[21]</sup> suggest utilizing linear mixing models for two point matches and models like the IsoSource model for more than three point matches. Models like IsoSource examine all possible combinations of each source contribution (0-100%) in small increments such as 1%. Feasible solutions are determined by combinations that sum to the detected mixture's isotopic signatures within a defined tolerance range (e.g. 0.1%). From these solutions the range and frequency of source contributions can be determined <sup>[33]</sup>. Zhao *et al.* <sup>[21]</sup> recommend the use of stable isotope analysis in concert with an

excavation method to determine rooting depth and biomass in the soil profile, as the ideal means to determine plant water source locations.

Stable isotopes found in water (hydrogen and oxygen) have also been used to study the water use efficiency of crops <sup>[34-39]</sup>. Within these works, improvements in water use efficiency are attributed to timing of water uptake by the crop (ex: during vegetative growth stage), as opposed to depth of use <sup>[34, 35]</sup>; that mild soil water depletion during seedling phase and severe soil water depletion during maturity phase can improve yields in limited irrigation settings <sup>[36]</sup>; that rain fed crops tend to have a low water use efficiency, and that this efficiency can be improved with irrigation inputs <sup>[37, 38]</sup>. These findings all point to the need for accurate and precise timing of irrigation depending on a crops specific need. Furthermore, they note that conserving water through prevention of both evaporation and deep percolation can improve water use efficiency <sup>[37, 39]</sup>. Some studies using stable isotopes to investigate irrigation water use efficiency are indicating that producers may be over-irrigating their crops <sup>[16, 22, 24]</sup>. The practice of deficit irrigation is common in regions that have low availability of irrigation water and in some cases it has been found that deficit irrigation during certain growth phases will result in physiological responses such as increased photosynthesis and carbon mobilization which significantly increase the yield and water use efficiency of wheat <sup>[40]</sup>. Interestingly, research has shown that maize will utilize shallow soil water during early growth stages, extend its water sourcing deeper through its vegetative growth, and then return to shallow water use at the end of its growth cycle <sup>[24]</sup>. Further to this point, Zhao *et al.* <sup>[21]</sup> found that maize utilizes the immobile soil water pool during its seedling phase, but as the roots grow to greater depth, it switches its water sourcing to the mobile soil water pool. More work is needed to determine if wheat and other cash crops exhibit similar behavior.

## **1.2 Plant Analyte Extraction Methods**

To access water stored in plants for stable isotope analysis, extraction methods are required. However, this extraction step is the bottleneck that limits our ability to process large numbers of samples on effective spatiotemporal scales <sup>[1]</sup>. A variety of field and laboratory-based methods exist for extracting plant analytes for stable isotope analysis. There are also direct equilibration

methods, which bypass the need for extraction, and show promise in allowing for higher spatial and temporal resolutions in regards to sample processing time <sup>[3, 4]</sup>. Laboratory based plant analyte extraction-analysis methods include: cryogenic vacuum distillation (CVD) <sup>[1, 41-43]</sup>; non-cryogenic batch method <sup>[44]</sup>; microwave extraction in-line analysis method <sup>[45]</sup>; azeotropic distillation <sup>[46]</sup>; centrifugation <sup>[2]</sup>; high pressure mechanical squeezing <sup>[47-49]</sup>; direct vapour equilibration (DVE) <sup>[50-52]</sup>. Field extraction-analysis methods include: direct vapour equilibration; in-situ monitoring <sup>[53, 54]</sup> and the Picarro Induction Module <sup>[55]</sup>. The term ‘extraction-analysis’ approach is used to encompass all affirmations systems as some systems directly analyze an extracted analyte, whereas others only extract an analyte, which must then be sent for further isotopic analysis.

### 1.3 Extraction Method Comparisons

While a variety of extraction-analysis approaches are available, little work has been done to compare the isotopic effects of these methods on the recovered analytes. The most commonly used plant analyte and soil water extraction method is the CVD method <sup>[3, 4]</sup>. Recently, Orlowski *et al.* <sup>[3]</sup> carried out the first formal inter-comparison of the major lab-based soil water extraction-analysis techniques. This study involved the extraction of spiked soil water from two unique soil types at three unique water contents per soil type. They found that the tested extraction-analysis methods returned result which were significantly different than the original spike water. The two methods which produced results closest to the spiked water signal were centrifugation and high-pressure mechanical squeezing, both of which are mechanical extraction methods (no phase change of water in extraction step).

Previously, studies have carried out a comparison of one extraction-analysis method to another; or carried out a comparison of a handful of methods <sup>[2, 44, 45, 52, 53, 56]</sup>. Typically, a newly developed extraction method would be compared to the ‘gold standard’ of CVD as a means of validating the new method. However, recent studies call into question the use of CVD as the ‘gold standard’, especially when extracting water from soils with low water contents or high clay content <sup>[3, 4, 8, 57]</sup>. The CVD system has been called into question because the method produces

results that are significantly different from all other methods, and from the spiked reference water for both  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  values [3].

*Sprenger et al.* [4] explain that the choice of soil water extraction method should be determined based on the compartment of the vadose zone the researcher wishes to access. Their work shows that each method of extraction can access different pools of soil water. Specifically, as one moves down this list of methods: lysimeters, zero tension lysimeters, wick samplers, suction lysimeters, mechanical squeezing, centrifugation, direct water vapour equilibration, distillation, cryogenic vacuum distillation; one is accessing more tightly bound water from finer pore sizes. Lysimeters, zero tension lysimeters, wick samplers and suction lysimeters, access the more mobile pools of soil water, leaving behind the more tightly bound water. High pressure mechanical squeezing and centrifugation access the more mobile pool, while also accessing some portion of the tightly bound pore water. Whereas, distillation and CVD are extracting 99.9% of the water from the soil (bulk soil water). The amount of tightly bound pore water accessed is dependent on the extraction conditions used in each system. A methodological comparison of plant water extraction techniques for stable isotope analysis was carried out by Yang *et al.* [6]. Within this review they detailed six methods of plant water extraction with emphasis on the fact that investigations into plant analyte stable isotopes are typically instigated to answer questions about plant water sourcing. Regarding extraction efficiencies, CVD and azeotropic distillation achieve up to 100% extraction efficiency, whereas, centrifugation and mechanical squeezing achieve extraction efficiencies in the range of 30-80%. Extraction efficiency with these two mechanical methods appears to be dependent on the water content of the sample, with lower water content samples resulting in lower extraction efficiencies [3]. For distillation type methods, incomplete extraction of water can result in Rayleigh type fractionation of the original water isotopic composition. For a detailed methodological review of the systems of soil water extraction-analysis the reader is directed to Sprenger *et al.* [4]. For a review of plant analyte extraction-analysis systems the reader is referred to Yang *et al.* [6]

#### 1.4 Plant Analyte Extraction and Stable Isotope Analysis Approach Problems

While there are advantages to using stable isotopes to determine plant water sourcing such as: small sample sizes, elimination of the need for radioactive tracers, estimates of water sourcing by depth, and the ability to study plant water uptake in natural settings <sup>[6]</sup>; there are also critical issues involved in their use. This approach often requires destructive sampling of the plant species in question and since the extraction process and subsequent isotope analysis is the current bottle neck, the spatio-temporal resolution of a study is controlled by the number of samples that can be processed in a given day via the chosen extraction-analysis method. These limitations make it impractical or impossible to collect samples over spatiotemporal scales that would allow for continuous monitoring of plant water use. Improvements to the portability of laser spectrometers <sup>[58]</sup> as well as improvements to direct equilibration methods <sup>[50, 51]</sup> and the development of in situ monitoring methods <sup>[53-55]</sup> are working to address this issue. Further research is required to better understand how the parameters used in the various extraction-analysis approaches affects the returned stable isotope results <sup>[3, 4, 7, 8]</sup>. Currently, there is no globally accepted protocol for extraction parameters (extraction temperature, pressure, duration etc.) used in each system, and the parameters used are not often reported in publications <sup>[8]</sup>. As such, Orlowski *et al.* <sup>[8]</sup> called for the inclusion of this information in all publications utilizing stable isotopes in their investigations; as their work showed that extraction parameters will have effects on the returned isotopic signals.

Another critical issue is that of co-extracted organic compounds, such as phenolic compounds and other xylem sap constituents <sup>[3, 59-61]</sup>. Typically, extracted plant analytes are analyzed via isotope ratio mass spectroscopy (IRMS) to reduce the errors associated with co-extracted organic compounds. However, the rise of low cost, rapid analysis by refractive laser based spectroscopy, known as isotope ratio infrared spectroscopy (IRIS), has led a number of groups to attempt to utilize this new analysis method on plant analytes <sup>[59]</sup>. Unfortunately, the frequencies at which the isotopes of water absorb light are similar to those of the problematic co-extracted compounds and thus results in errors in the measurement of  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  values on refractive laser spectrometers <sup>[3, 4, 59, 62]</sup>. This problem also extends to the microwave extraction system <sup>[45]</sup> and the DVE system as they utilize continuous-flow laser spectroscopy. Thus, any co-extracted

organics present in the headspace of analyzed samples will potentially result in spectral contamination during their real-time analysis. It is also important to note that while the band of the electromagnetic spectrum where water absorbance is measured is relatively large, the two major manufacturers of IRIS systems Picarro (Picarro, Santa Clara, CA, USA) and Los Gatos Research (LGR Inc., San Jose, CA, USA) utilize only small portions of that band for their isotopic measurements. The details regarding the portion of the electromagnetic spectrum water absorbance band being used by each company, and the mathematical fitting models used to derive the isotopic composition of the measured water are proprietary information and thus difficult to investigate by researchers. Some groups have attempted to use pre-processing filtration and post-processing corrections to address this issue <sup>[60, 61, 63]</sup>, but have met with varying amounts of success. More work is still needed to address this problem.

Research has also shown that spiked soil water trials, used to investigate methodological effects on returned soil water isotope signals, may be problematic <sup>[4, 57, 64]</sup>. These spike trials tend to return results which deviate from the original spiked signal, and this issue becomes more pronounced with increasing clay content and decreasing water content <sup>[3, 4, 8]</sup>. Clay interlayer absorption of water (crystalline water) and drying temperatures used in spike trial preparation may be responsible for these issues <sup>[4, 57, 64]</sup>. Finally, it has been noted that there is, as of yet, no method for defining a ‘reference’ signal for plant water in studies comparing plant water extraction method accuracy, so our ability to apply Z-score <sup>[65]</sup> style accuracy analysis is limited in plant water extraction-analysis method inter-comparison studies <sup>[2, 52]</sup>.

### **1.5 Ecohydrological Separation in Plant Water Use**

The finding that precipitation, ground, and stream water isotopic signals differed from bulk soil water and vegetation isotopic signals by Brooks *et al.* <sup>[9]</sup> led to the development of the ‘two water worlds’ hypothesis, also known as ecohydrological separation. Ecohydrological separation, was first discovered by comparing the results of extracted soil pore water collected by suction lysimeters (mobile soil water fraction) and CVD (bulk soil water fraction) with collected plant analyte samples (also extracted via CVD). It was found that when the results were plotted in dual isotope space [ $\delta^{18}\text{O}$  vs  $\delta^2\text{H}$ ], that the mobile soil water, extracted by suction lysimeters, plotted

along the local meteoric water line (LMWL), while the bulk soil water, extracted cryogenically, plotted along an evaporative line, below the LMWL. Plant analyte data from the same location also plotted along the evaporative soil line, implying that plants were preferentially using the more tightly bound soil water. Initially the phenomenon of ecohydrological separation was found to be present in Mediterranean climates where the precipitation input phase (rainy season) was uncoordinated with the main plant transpiration phase [7, 9, 66].

Further work studied this phenomenon in regions where rainfall was less seasonally variable and where precipitation inputs are coordinated with primary productivity [67, 68]. Geris *et al.* [68] did not find clear evidence of ecohydrological separation in wet, low energy environments (Northern Scotland); but admits that more work is needed to confirm this. Evaristo *et al.* [67] found evidence of ecohydrological separation in the in-phase environment they studied (Puerto Rico), but suggested that this may be a result of the mechanisms which result in isotopic enrichment of soil water as opposed to the synchronicity of precipitation inputs and primary productivity. Further work in the form of a meta-analysis of 47 sites across the world showed that ecohydrological separation is common in many ecosystems [69]. In addition, remote sensing work attempting to establish a baseline for global deuterium (HDO) budgets found that the hydrological disconnect between mobile and immobile water pools was both widespread and considerable at global scales [70, 71]. The mechanism(s) responsible for ecohydrological separation are still being investigated. Originally, Brooks *et al.* [9] suggested the following mechanism: during wet up, the small pore spaces are filled with water that becomes isotopically depleted with depth. Then, during the rainy months, water moves preferentially through the macro pore space within the soil, recharging ground water and connecting to stream channels, with little interaction between the water moving through the preferential flow paths and that of the tightly bound water filled during wet up. Finally, during the dry months, as the macro pores drain, the trees preferentially access the more tightly bound, isotopically depleted, micro pore spaces. Sprenger *et al.* [72] suggests an alternative mechanism, wherein, mixing of enriched soil water with that of unfractionated precipitation results in a diminishing evaporative fractionation signal with increasing depth until the percolating water's isotopic signal merges with the LMWL. Review work by Berry *et al.* [73] proposes multiple alternative hypothesis to explain TWWH style



behavior, the reader is referred there for further detail. More research is needed to understand the mechanisms responsible for development the TWWH style soil water isotopic composition. Furthermore, better understanding of the effects that methods of extraction-analysis are having on returned stable isotopes results may help us better understand this phenomenon

### 1.6 Research Gap

While a variety of extraction-analysis approaches exist to access plant water for stable isotope analysis, the comparability and accuracy of those methods are still in need of further definition. McDonnell <sup>[7]</sup>, Sprenger *et al.* <sup>[4]</sup> and Orlowski *et al.* <sup>[8]</sup> have all called for a comprehensive inter-comparison of the systems used in extracting plant analytes and soil pore water. As such, Orlowski *et al.* <sup>[3]</sup> showed, through an inter-method comparison of five soil pore water extraction techniques, that soil water extraction systems should not be considered interchangeable. An inter-method comparison checking the comparability and accuracy of the systems used to extract bulk plant analytes is still needed. It has been noted that there is no method for obtaining a ‘reference’ signal for plant water in studies examining plant analyte extraction method accuracy <sup>[2, 52]</sup>. However, work by Phillips and Gregg <sup>[33]</sup> and Zhao *et al.* <sup>[21]</sup> has made progress in the direction of determining a source water reference value for plant water sourcing through the multi-source model IsoSource.

The recently discovered phenomenon of ecohydrological separation has been shown to be present across a wide variety of ecosystems <sup>[69]</sup>. The mechanisms that controls this behavior are still being debated, with one mechanism detailed by the researchers that discovered the phenomenon <sup>[9]</sup>, another recently suggested by Sprenger *et al.* <sup>[72]</sup> and multiple alternatives proposed by Berry *et al.* <sup>[73]</sup>. Research by Orlowski *et al.* <sup>[3]</sup> and Gaj *et al.* <sup>[64]</sup> have shown that artifacts from the systems used to extract water from soil have the capacity to create the observed phenomenon of ecohydrological separation by modifying returned isotopic results.

Furthermore, the discovery by Zhao *et al.* <sup>[74]</sup> and Vargas *et al.* <sup>[75]</sup> that significant amounts of deuterium fractionation between plant tissue and source water may be occurring during uptake or within the plant itself has implications for the use of mixing models in determining plant source water, and the assumptions underlaying stable isotope approach in general. New work has shed

light on plant water use and sharing strategies <sup>[76, 77]</sup> which could be causing modifications to stable isotope composition during water uptake and within the plant <sup>[74, 75, 78]</sup>. Additionally, hydraulic redistribution of water in the soil both vertically and laterally <sup>[73, 79-85]</sup>, and the use of atmospheric water during foliar uptake (Dawson 1998; Limm *et al.* 2009; Eller *et al.* 2013; Berry *et al.* 2014; Earles *et al.* 2016) are shedding light on alternative plant water sources, whose isotopic signals are sub-components of the bulk plant water collected during extraction. In light of these new findings, the assumptions underlying the stable isotope approach set down in 1995 by Brunel *et al.* <sup>[86]</sup> are in urgent need of re-assessment. Finally, there is currently no specified standard operating procedure for the stable isotope approach nor for the various methods of plant and soil water extraction. This should be addressed in order to bring a standard approach to the fields utilizing these techniques.

### **1.7 Objectives and Hypotheses**

The purpose of this thesis is twofold: First to inter-compare the available extraction-analysis systems used to extract plant analyte for stable isotope analysis and determine if they produce accurate, interchangeable results. Second, to quantify the effects of two commonly cited organic contaminants (methanol and ethanol) on the IRIS analysis system: off-axis integrated cavity output spectroscopy (OA-ICOS), and to test an approach for their detection during vapour mode analysis. The results of this work will have important implications for fields that utilize plant water stable isotope  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  data.

Through a series of controlled experiments using both lab and field-based data collection methods, and through review of current literature, the following objectives will be met:

1. Inter-compare the methods of plant water extraction for stable isotope analysis of  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  values to determine if these extraction-analysis systems return accurate, interchangeable results.
2. a. Examine how the presence of co-extracted organic contaminants like methanol and ethanol in plant analyte samples affects stable isotope results returned by the Los Gatos Research OA-ICOS.

b. Assess the effectiveness of the Absorption plot and the Spectra Fit Residuals plot at detecting organic contamination during vapour-mode analysis via OA-ICOS.

The main objective in Chapter 2 is to inter-compare six lab-based plant water extraction-analysis systems. While CVD is the most commonly utilized extraction system <sup>[3, 4]</sup>, I tested the following extraction-analysis methods: direct vapour equilibration (DVE) <sup>[50, 87]</sup>, high pressure mechanical squeezing (HPMS) <sup>[48]</sup>; centrifugation <sup>[2]</sup>; cryogenic vacuum distillation in two forms based on: a) Orłowski *et al.* <sup>[43]</sup> (hereafter CVD-1) and b) Koeniger *et al.* <sup>[41]</sup> (hereafter CVD-2); microwave extraction off-axis integrated cavity output spectroscopy (ME-OA-ICOS) analysis method, based on Munksgaard *et al.* <sup>[45]</sup>. I use the term extraction-analysis system to indicate that certain methods utilize in-line analysis via direct connection to an isotope analyzer as opposed to extracting liquid water or analyte for later analysis. I pose the null hypothesis that all systems of extraction-analysis will return water that when analyzed for its isotopic composition will provide statistically similar results. To carry out this test, spring wheat was grown under controlled conditions with water inputs of known isotopic composition. Spring wheat was chosen for the ease of growing it under controlled conditions, for its rapidity of growth, and for its economic value as a globally important cash crop. The spring wheat was split into four plant parts: the head, leaf, stem, and root crown components, replicated, and run through the extraction-analysis methods. When possible, and where liquid analyte was collected, it was analyzed via IRMS and IRIS approaches to quantify any analytical differences. The specific questions addressed in this work are: How do the returned stable isotope results compare between the different methods of extraction? Do the co-extracted volatile organic compounds (methanol and ethanol) appear to affect the returned isotopic results? Which extraction method has the best sample purity, repeatability, and throughput? This study was carried out over the summer of 2016, was submitted for peer review in December 2017, and was accepted for publication in March 2018 [citation: Millar C, Pratt D, Schneider D J, McDonnell J J. A comparison of extraction systems for plant water stable isotope analysis. *Rapid Commun Mass Sp*; **32**(13):1031-1044 2018].

The focus of Chapter 3 was to further investigate potential method differences between the DVE approach and the CVD-2 approach. The findings of Chapter 2 implied that there were not

significant differences between the stable isotope results returned by these two systems. However, relatively large volumes of co-extracted methanol and ethanol were present in the analyte produced by the CVD-2 system in Chapter 2. Due to the small sample sizes used for comparison between the tested methods in Chapter 2, our statistical power was relatively weak. We sought to improve our understanding of potential differences in isotopic results returned by the DVE and CVD-2 systems by carrying out a follow up study. Spring wheat was again grown under controlled conditions and was sampled over an 8-week period, collecting samples for extraction and analysis on both the DVE and CVD-2 systems. The null hypothesis was that both systems would produce plant analyte that when analyzed would have isotopically similar results. This study was carried out in the summer of 2017. A manuscript is in preparation for publication.

The focus in Chapter 4 is on the effects of methanol (MeOH) and ethanol (EtOH) contamination in water samples when analyzed via the IRIS method: off axis integrated cavity output spectroscopy (OA-ICOS). This was carried out in response to the findings of Chapter 2, that indeed the presence of organic contaminants in plant extracted analytes appeared to impact isotopic results returned by the OA-ICOS system. Analytes extracted from plants for stable isotope analysis commonly contain co-extracted organic contaminants in addition to the extracted water. These organic contaminants are known to cause substantial errors in returned isotopic results when that water is analyzed via IRIS methods <sup>[60, 62, 63, 88]</sup>. Therefore, plant extracted analytes are typically analyzed by IRMS methods wherein the size of the contamination effect is always directly related to the concentration of the contaminant in the sample and its isotopic ratio, and errors are therefore correctable <sup>[62]</sup>. However, with the rise of low cost IRIS analysis approaches, as well as the development of the DVE method <sup>[50, 87]</sup> that utilizes real-time in-line analysis via OA-ICOS, and in-situ analysis approaches <sup>[53]</sup>, quantifying the effects of MeOH and EtOH contamination on the OA-ICOS system in liquid and vapour analysis modes are of interest. Previous work notes issues with quantifying the effects of MeOH and EtOH contamination when analyzed via the Picarro (Picarro, Santa Clara, CA, USA) cavity ring down spectroscopy (CRDS) method <sup>[62]</sup>. Research has also shown that CRDS and OA-ICOS approaches produced stable isotope results that skewed in opposite (positive vs negative) directions in the presence of organic contamination <sup>[88]</sup>. Spectral contamination identification

software has been developed by Los Gatos Research (Los Gatos Research Inc., San Jose, CA, USA), the producer of the OA-ICOS analysis system and its effectiveness was assessed by [63]. However, no such detection software exists for use during vapour-mode analysis via OA-ICOS. As such, we were interested in the use of the ‘Absorption plot’ and ‘Spectra Fit Residuals plot’, sub components of the OA-ICOS system, as tools for flagging contaminated samples during vapour-mode analysis on LGR’s OA-ICOS systems. MeOH-water and EtOH-water mixtures were created and subsequently analyzed in liquid and vapour mode on the OA-ICOS system. These isotopic results were then compared to liquid water analyses results produced via IRMS, utilizing replicates of the same contaminant-water samples. The experiment was carried out in the fall of 2017 and the manuscript was prepared in January-February 2018. This manuscript is in production for publication.

## 1.8 References

- [1] West A G, Patrickson S J, Ehleringer J R. Water extraction times for plant and soil materials used in stable isotope analysis. *Rapid Commun Mass Sp* 2006; 20(8):1317-1321.
- [2] Peters L I, Yakir D. A direct and rapid leaf water extraction method for isotopic analysis. *Rapid Commun Mass Sp* 2008; 22(18):2929-2936.
- [3] Orłowski N, Pratt D L, McDonnell J J. Intercomparison of soil pore water extraction methods for stable isotope analysis. *Hydrol Process* 2016b; 30(19):3434-3449.
- [4] Sprenger M, Herbstritt B, Weiler M. Established methods and new opportunities for pore water stable isotope analysis. *Hydrol Process* 2015; 29(25):5174-5192.
- [5] Dawson T E, Ehleringer J R. Stream side trees that do not use stream water. *Nature* 1991; 350(335-337).
- [6] Yang Q, Xiao H, Zhao L, Zhou M, Li C, Cao S. Stable isotope techniques in plant water sources a review. *Sciences in Cold and Arid Regions* 2010; 2(2):0112-0122.
- [7] McDonnell J J. The two water worlds hypothesis ecohydrological separation of water between trees and streams. *WIREs: Water* 2014; 1(4):323-329.
- [8] Orłowski N, Breuer L, McDonnell J J. Critical issues with cryogenic extraction of soil water for stable isotope analysis. *Ecohydrology* 2016a; 9(1):1-5.
- [9] Brooks J R, Barnard H R, Coulombe R, McDonnell J J. Ecohydrologic separation of water between trees and streams in a mediterranean climate. *Nature Geoscience* 2010; 3(2):100-104.
- [10] Phillips F M. Hydrology: Soil-water bypass. *Nature Geoscience* 2010; 3(2):77-78.
- [11] Ehleringer J R, Dawson T E. Water uptake by plants: Perspectives from stable isotope composition. *Plant, Cell and Environment* 1992; 15(9):1073-1082.
- [12] Williams D G, Ehleringer J R. Intra- and interspecific variation for summer precipitation use in pinyon-juniper woodlands. *Ecological Monographs* 2000; 70(4):517-537.

- [13] MeiBner M, Kohler M, Schwendenmann L, Holscher D, Dycksmans J. Soil water uptake by trees using water stable isotopes ( $\delta^2\text{H}$  and  $\delta^{18}\text{O}$ )—a method test regarding soil moisture, texture and carbonate. *Plant and Soil* 2013; 376(1):327-335.
- [14] Schwinning S, Davis K, Richardson L, Ehleringer J. Deuterium enriched irrigation indicates different forms of rain use in shrub/grass species of the colorado plateau. *Oecologia* 2002; 130(3):345-355.
- [15] Rothfuss Y, Biron P, Braud I, Canale L, Durand J-L, Gaudet J-P, Richard P, Vauclin M, Bariac T. Partitioning evapotranspiration fluxes into soil evaporation and plant transpiration using water stable isotopes under controlled conditions. *Hydrological Processes* 2010; 24(22):3177-3194.
- [16] Wang P, Song X, Han D, Zhang Y, Zhang B. Determination of evaporation, transpiration and deep percolation of summer corn and winter wheat after irrigation. *Agricultural Water Management* 2012; 105(32-37).
- [17] Dubbert M, Piayda A, Cuntz M, Correia A C, Costa E S F, Pereira J S, Werner C. Stable oxygen isotope and flux partitioning demonstrates understory of an oak savanna contributes up to half of ecosystem carbon and water exchange. *Front Plant Sci* 2014; 5(530).
- [18] Isaac M E, Anglauer L C. An in situ approach to detect tree root ecology: Linking ground-penetrating radar imaging to isotope-derived water acquisition zones. *Ecol Evol* 2013; 3(5):1330-1339.
- [19] Rossatto D R, de Carvalho Ramos Silva L, Villalobos-Vega R, Sternberg L d S L, Franco A C. Depth of water uptake in woody plants relates to groundwater level and vegetation structure along a topographic gradient in a neotropical savanna. *Environmental and Experimental Botany* 2012; 77(259-266).
- [20] Bachmann D, Gockele A, Ravenek J M, Roscher C, Strecker T, Weigelt A, Buchmann N. No evidence of complementary water use along a plant species richness gradient in temperate experimental grasslands. *PLoS One* 2015; 10(1):e0116367.
- [21] Zhao P, Tang X, Zhao P, Tang J. Dynamics of water uptake by maize on sloping farmland in a shallow entisol in southwest china. *Catena* 2016b; 147(511-521).
- [22] Lia Q, Donga B, Qiao Y, Liu M, Zhang J. Root growth, available soil water, and water-use efficiency of winter wheat under different irrigation regimes applied at different growth stages in north china. *Agricultural Water Management* 2010; 97(10):1676-1682.
- [23] Qiu G Y, Wang L, He X, Zhang X, Chen S, Chen J, Yang Y. Water use efficiency and evapotranspiration of winter wheat and its response to irrigation regime in the north china plain. *Agricultural and Forest Meteorology* 2008; 148(11):1848-1849.
- [24] Wang P, Song X, Han D, Zhang Y, Liu X. A study of root water uptake of crops indicated by hydrogen and oxygen stable isotopes a case in shanxi province, china. *Agricultural Water Management* 2010; 97(3):475-482.
- [25] UNFAO, in *FAO Plant Production and Protection Series, Vol. 30* (Eds.: B. C. Curtis, S. Rajaram, H. Gómez Macpherson), FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS, Rome, **2002**.
- [26] Vörösmarty C J, Green P, Salisbury J, Lammers R B. Global water resources: Vulnerability from climate change and population growth. *Science* 2000; 289(5477):284-288.

- [27] Schewe J, Heinke J, Gerten D, Haddeland I, Arnell N W, Clark D B, Dankers R, Eisner S, Fekete B M, Colon-Gonzalez F J, Gosling S N, Kim H, Liu X, Masaki Y, Portmann F T, Satoh Y, Stacke T, Tang Q, Wada Y, Wisser D, Albrecht T, Frieler K, Piontek F, Warszawski L, Kabat P. Multimodel assessment of water scarcity under climate change. *Proc Natl Acad Sci U S A* 2014; 111(9):3245-3250.
- [28] Oki T, Kanae S. Global hydrological cycles and world water resources. *Science* 2006; 313(5790):1068-1072.
- [29] Hanjra M A, Qureshi M E. Global water crisis and future food security in an era of climate change. *Food Policy* 2010; 35(5):365-377.
- [30] Dawson T E, Pate J S. Seasonal water uptake and movement in root systems of australian phraeatophytic plants of dimorphic root morphology a stable isotope investigation. *Oecologia* 1996; 107(1):13-20.
- [31] Thorburn P J, Ehleringer J R. Root water uptake of field-growing plants indicated by measurements of natural-abundance deuterium. *Plant and Soil* 1995; 177(2):225-233.
- [32] Brunel J-P, Walker G R, Kennett-Smith A K. Field validation of isotopic procedures for determining sources of water used by plants in a semi-arid environment. *Journal of Hydrology* 1995; 167(1-4):351-368.
- [33] Phillips D L, Gregg J W. Source partitioning using stable isotopes: Coping with too many sources. *Oecologia* 2003; 136(2):261-269.
- [34] Entz M H, Gross K G, Fowler D B. Root growth and soil water extraction by winter and spring wheat. *Canadian Journal of Plant Science* 1991; 72(4):1109-1120.
- [35] Angus J F, van Herwaarden A F. Increasing water use and water use efficiency in dryland wheat. *Agronomy Journal* 2001; 93(2):290-298.
- [36] Kang S, Zhang L, Liang Y, Hu X, Cai H, Gu B. Effects of limited irrigation on yield and water use efficiency of winter wheat in the loess plateau of china. *Agricultural Water Management* 2002; 55(3):203-216.
- [37] Huang Y, Chen L, Fu B, Huang Z, Gong J. The wheat yields and water-use efficiency in the loess plateau: Straw mulch and irrigation effects. *Agricultural Water Management* 2005; 72(3):209-222.
- [38] Sadras V O, Angus J F. Benchmarking water-use efficiency of rainfed wheat in dry environments. *Crop and Pasture Science* 2005; 57(8):847-856.
- [39] Deng X-P, Shan L, Zhang H, Turner N C. Improving agricultural water use efficiency in arid and semiarid areas of china. *Agricultural Water Management* 2006; 80(1-3)
- [40] Xue Q, Zhu Z, Musick J T, Stewart B A, Dusekb D A. Physiological mechanisms contributing to the increased water-use efficiency in winter wheat under deficit irrigation. *Journal of Plant Physiology* 2006; 163(2):154-164.
- [41] Koeniger P, Marshall J D, Link T, Mulch A. An inexpensive, fast, and reliable method for vacuum extraction of soil and plant water for stable isotope analyses by mass spectrometry. *Rapid Commun Mass Sp* 2011; 25(20):3041-3048.
- [42] Goebel T S, Lascano R J. System for high throughput water extraction from soil material for stable isotope analysis of water. *Journal of Analytical Sciences, Methods and Instrumentation* 2012; 02(04):203-207.
- [43] Orłowski N, Frede H G, Brüggemann N, Breuer L. Validation and application of a cryogenic vacuum extraction system for soil and plant water extraction for isotope analysis. *J Sensors* 2013; 2(2):179-193.

- [44] Vendramini P F, Sternberg L d S L. A faster plant stem-water extraction method. *Rapid Commun Mass Sp* 2008; 21(2):164-168.
- [45] Munksgaard N C, Cheesman A W, Wurster C M, Cernusak L A, Bird M I. Microwave extraction-isotope ratio infrared spectroscopy (me-iris): A novel technique for rapid extraction and in-line analysis of  $\delta^{18}\text{O}$  and  $\delta^2\text{H}$  values of water in plants, soils and insects. *Rapid Commun Mass Sp* 2014; 28(20):2151-2161.
- [46] Revesz K, Woods P H. A method to extract soil water for stable isotope analysis. *Journal of Hydrology* 1990; 115(1-4):397-406.
- [47] White J W C, Cook E R, Lawrence J R, Broecker W S. The  $\delta\text{H}$  ratios of sap in trees implications for water sources and tree ring  $\delta\text{H}$  ratios. *Geochimica et Cosmochimica Acta* 1985; 49(1):237-246.
- [48] Bottcher G, Brumsack H J, Heinrichs H, Pohlmann M. A new high pressure squeezing technique for pore fluid extraction from terrestrial soils. *Water Air Soil Poll* 1997; 94(3):289-296.
- [49] Kelln C J, Wassenaar L I, Hendry M J. Stable isotopes ( $\delta^{18}\text{O}$ ,  $\delta^2\text{H}$ ) of pore waters in clay-rich aquitards: A comparison and evaluation of measurement techniques. *Groundwater Monitoring and Remediation* 2001; 21(2):108-116.
- [50] Hendry M J, Schmeling E, Wassenaar L I, Barbour S L, Pratt D. Determining the stable isotope composition of pore water from saturated and unsaturated zone core: Improvements to the direct vapour equilibration laser spectrometry method. *Hydrol Earth Syst Sc* 2015; 19(11):4427-4440.
- [51] Pratt D L, Lu M, Barbour S L, Hendry M J. An evaluation of materials and methods for vapour measurement of the isotopic composition of pore water in deep, unsaturated zones. *Isotopes in Environmental and Health Studies* 2016; 52(4-5):529-543.
- [52] Scrimgeour C M. Measurement of plant and soil water isotope composition by direct equilibration methods. *J Hydrology* 1995; 172(1-4):261-274.
- [53] Volkmann T H M, Kühnhammer K, Herbstritt B, Gessler A, Weile M. A method for in situ monitoring of the isotope composition of tree xylem water using laser spectroscopy. *Plant Cell Environ* 2016; 39(9):2055-2063.
- [54] Volkmann T H M, Weiler M. Continual in situ monitoring of pore water stable isotopes in the subsurface. *Hydrology and Earth System Sciences* 2014; 18(5):1819-1833.
- [55] Picarro, **2016**.
- [56] Figueroa-Johnson M A, Tindall J A, Friedel M. A comparison of  $\delta^{18}\text{O}$  composition of water extracted from suction lysimeters, centrifugation, and azeotropic distillation. *Water Air Soil Poll* 2007; 184(1-4):63-75.
- [57] Gaj M, Kaufhold S, McDonnell J J. Potential limitation of cryogenic vacuum extractions and spiked experiments. *Rapid Commun Mass Spectrom* 2017b; 31(9):821-823.
- [58] LGR, Vol. 2018, **2018**.
- [59] Leen J B, Berman E S F, Liebson L, Gupta M. Spectral contaminant identifier for off-axis integrated cavity output spectroscopy measurements of liquid water isotopes. *Rev Sci Instrum* 2012; 83(4)
- [60] Martin-Gomez P, Barbeta A, Voltas J, Penuelas J, Dennis K, Palacio S, Dawson T E, Ferrio J P. Isotope-ratio infrared spectroscopy: A reliable tool for the investigation of plant-water sources? *New Phytol* 2015; 207(3):914-927.



- [61] Hendry M J, Richman B, Wassenaar L I. Correcting for methane interferences on  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  measurements in pore water using  $\text{H}_2\text{O}$  liquid- $\text{H}_2\text{O}$  vapor equilibration laser spectroscopy. *Anal Chem* 2011; 83(14):5789-5796.
- [62] Brand W A, Geilmann H, Crosson E R, Rella C W. Cavity ring-down spectroscopy versus high-temperature conversion isotope ratio mass spectrometry; a case study on  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  of pure water samples and alcohol/water mixtures. *Rapid Commun Mass Sp* 2009; 23(12):1879-1884.
- [63] Schultz N M, Griffis T J, Lee X, Baker J M. Identification and correction of spectral contamination in  $2\text{H}/1\text{H}$  and  $18\text{O}/16\text{O}$  measured in leaf, stem, and soil water. *Rapid Commun Mass Spectrom* 2011; 25(21):3360-3368.
- [64] Gaj M, Kaufhold S, Koeniger P, Beyer M, Weiler M, Himmelsbach T. Mineral mediated isotope fractionation of soil water. *Rapid Commun Mass Spectrom* 2017a; 31(3):269-280.
- [65] Wassenaar L I, Ahmad M, Aggarwal P, van Duren M, Poltenstein L, Araguas L, Kurtas T. Worldwide proficiency test for routine analysis of  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  in water by isotope-ratio mass spectrometry and laser absorption spectroscopy. *Rapid Commun Mass Sp* 2012; 26(15):1641-1648.
- [66] Goldsmith G R, Muñoz-Villers L E, Holwerda F, McDonnell J J, Asbjornsen H, Dawson T E. Stable isotopes reveal linkages among ecohydrological processes in a seasonally dry tropical montane cloud forest. *Ecohydrology* 2012; 5(6):779-790.
- [67] Evaristo J, McDonnell J J, Scholl M A, Bruijnzeel L A, Chun K P. Insights into plant water uptake from xylem-water isotope measurements in two tropical catchments with contrasting moisture conditions. *Hydrological Processes* 2016; 30(18):3210-3227.
- [68] Geris J, Tetzlaff D, McDonnell J, Anderson J, Paton G, Soulsby C. Ecohydrological separation in wet, low energy northern environments? A preliminary assessment using different soil water extraction techniques. *Hydrological Processes* 2015; 29(25):5139-5152.
- [69] Evaristo J, Jasechko S, McDonnell J J. Global separation of plant transpiration from groundwater and streamflow. *Nature* 2015; 525(7567):91-94.
- [70] Good S P, Noone D, Bowen G J. Hydrologic connectivity constrains partitioning of global terrestrial water fluxes. *Science* 2015a; 349(6244):175-177.
- [71] Good S P, Noone D, Kurita N, Benetti M, Bowen G J.  $\delta^2\text{H}$  isotope ratios in the global hydrologic cycle. *Geophysical Research Letters* 2015b; 42(12):5042-5050.
- [72] Sprenger M, Leistert H, Gimbel K, Weiler M. Illuminating hydrological processes at the soil-vegetation-atmosphere interface with water stable isotopes. *Reviews of Geophysics* 2016a; 54(3):674-704.
- [73] Berry Z C, Evaristo J, Moore G, Poca M, Steppe K, Verrot L, Asbjornsen H, Borma L S, Bretfeld M, Hervé-Fernández P, Seyfried M, Schwendenmann L, Sinacore K, De Wispelaere L, McDonnell J. The two water worlds hypothesis: Addressing multiple working hypotheses and proposing a way forward. *Ecohydrology* 2017:e1843.
- [74] Zhao L, Wang L, Cernusak L A, Liu X, Xiao H, Zhou M, Zhang S. Significant difference in hydrogen isotope composition between xylem and tissue water in *Populus euphratica*. *Plant Cell Environ* 2016a; 39(8):1848-1857.
- [75] Vargas A I, Schaffer B, Yuhong L, Sternberg L d S L. Testing plant use of mobile vs immobile soil water sources using stable isotope experiments. *New Phytol* 2017; 215(2):582-594.

- [76] Simard S W, Perry D A, Jones M D, Myrold D D, Durall D M, Molina R. Net transfer of carbon between ectomycorrhizal tree species in the field. *Nature* 1997; 388(579-582).
- [77] Pickles D R B, Simard S W, in *Mycorrhizal mediation of soil – fertility, structure, and carbon storage*. (Eds.: N. C. Johnson, C. Gehring, J. Jansa), Elsevier, Amsterdam, **2017**, pp. 319-339. .
- [78] Cernusak L A, Barbour M M, Arndt S K, Cheesman A W, English N B, Feild T S, Helliker B R, Holloway-Phillips M M, Holtum J A, Kahmen A, McInerney F A, Munksgaard N C, Simonin K A, Song X, Stuart-Williams H, West J B, Farquhar G D. Stable isotopes in leaf water of terrestrial plants. *Plant Cell Environ* 2016; 39(5):1087-1102.
- [79] Domec J C, King J S, Nooemets A, Treasure E, Gavazzi M J, Sun G, McNulty S G. Hydraulic redistribution of soil water by roots affects whole-plant evapotranspiration and net ecosystem carbon exchange. *New Phytologist*, 2010; 187(1):187, 171–183.
- [80] Prieto I, Armas C, Pugnaire F I. Water release through plant roots: New insights into its consequences at the plant and ecosystem level. *New Phytologist* 2012; 193(4):830-841.
- [81] Sardans J, Peñuelas J. Hydraulic redistribution by plants and nutrient stoichiometry: Shifts under global change. *Ecohydrology* 2014; 7(1):1-20.
- [82] Richards J H, Caldwell M M. Hydraulic lift: Substantial nocturnal water transport between soil layers by artemisia tridentata roots. *Oecologia* 1987; 73(4):486-489.
- [83] Emerman S H, Dawson T E. Hydraulic lift and its influence on the water content of the rhizosphere: An example from sugarmaple, acer saccharum. *Oecologia* 1996; 108(2):273-278.
- [84] Kurz-Besson C, Otieno D, Lobo do Vale R, Siegwolf R, Schmidt M, Herd A, Nogueira C, Soares David T, Soares David J, Tenhunen J, Pereira J S, Chaves M. Hydraulic lift in cork oak trees in a savannah-type mediterranean ecosystem and its contribution to the local water balance. *Plant Soil* 2006; 282(1-2):361-378.
- [85] Brooks J R, Meinzer F C, Warren J M, Domec J-C, Coulombe R. Hydraulic redistribution in a douglas-fir forest: Lessons from system manipulations. *Plant, Cell & Environment*. 2006; 29(1):138-150.
- [86] Brunel J P, Walker G R, Kennett-Smith A K. Field validation of isotopic procedures for determining sources of water used by plants in a semi-arid environment. *Journal of Hydrology* 1995; 167(1-4):351-368.
- [87] Wassenaar L I, Hendry M J, Chostner V L, G.P. L. High resolution pore water  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  measurements by  $\text{H}_2\text{O}(\text{liquid})\text{-H}_2\text{O}(\text{vapor})$  equilibration laser spectroscopy. *Envir Sci Tech* 2008; 42(24):9262-9267.
- [88] West A G, Goldsmith G R, Brooks P D, Dawson T E. Discrepancies between isotope ratio infrared spectroscopy and isotope ratio mass spectrometry for the stable isotope analysis of plant and soil waters. *Rapid Commun Mass Sp* 2010; 24(14):1948-1954.

## Chapter 2: A Comparison of Extraction Systems for Plant Water Stable Isotope Analysis.

Cody Millar <sup>1</sup>, Dyan Pratt <sup>1</sup>, David J. Schneider <sup>2</sup>, and Jeffrey J. McDonnell <sup>1</sup>.

1. Global Institute for Water Security, School of Environment and Sustainability, University of Saskatchewan, 11 Innovation Boulevard, Saskatoon, SK, S7N 3H5, Canada
2. Global Institute for Food Security, University of Saskatchewan, 110 Gymnasium Place, Saskatoon, SK, S7N 4J8, Canada.

Status: Published

Citation: Millar C, Pratt D, Schneider D J, McDonnell J J. (2018) A comparison of extraction systems for plant water stable isotope analysis. *Rapid Commun Mass Sp*; 32(13): 1031-1044.

### 2.1 Abstract

**RATIONALE:** The stable isotopes of water ( $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  values) have been widely used to trace water in plants in a variety of physiological, ecohydrological, biogeochemical and hydrological studies. In such work, the analyte must first be extracted from samples, prior to isotopic analysis. While cryogenic vacuum distillation is currently the most widely used method in the literature, a variety of extraction-collection-analysis methods exist. A formal inter-method comparison on plant tissues has yet to be carried out.

**METHODS:** We perform an inter-method comparison of six plant water extraction techniques: direct vapour equilibration, microwave extraction, two unique versions of cryogenic vacuum distillation, centrifugation, and high-pressure mechanical squeezing. These methods were applied to four isotopically unique plant portions (head, stem, leaf, and root crown) of spring wheat (*Triticum aestivum* L.). Extracted plant water was analyzed via spectrometric (OA-ICOS) and mass based (IRMS) analysis systems when possible. Spring wheat was grown under controlled conditions with irrigation inputs of a known isotopic composition.

**RESULTS:** Results indicate that the methods of extraction return markedly different isotopic signatures. Centrifugation, microwave extraction, direct vapour equilibration, and high-pressure

mechanical squeezing returned results more enriched in  $^2\text{H}$  and  $^{18}\text{O}$ . Both cryogenic vacuum distillation systems, and the high-pressure mechanical squeezing method returned results more depleted in  $^2\text{H}$  and  $^{18}\text{O}$ , depending upon the plant portion extracted. The various methods also produced differing concentrations of co-extracted organic compounds related to the mode of extraction. Overall, the direct vapour equilibration method outperformed all other methods.

**CONCLUSIONS:** Despite its popularity, cryogenic vacuum distillation was outperformed by the direct vapour equilibration method in terms of limited co-extraction of volatile organic compounds, rapid sample throughput, and near instantaneous returned stable isotope results. More research is now needed with other plant species, especially woody plants, to see how far the findings from this study could be extended.

## 2.2 Introduction

The stable isotopes of water ( $^2\text{H}/^1\text{H}$  and  $^{18}\text{O}/^{16}\text{O}$  ratios) have been widely used to trace water in a variety of ecohydrological, biogeochemical and climatological studies <sup>[1-4]</sup>. While many of these applications sample liquid water from streams and lakes, and relate it to precipitation, increasing numbers of studies trace plant water sources. In such work, analytes must first be extracted from the plant tissue. Cryogenic vacuum distillation (CVD), is considered the standard method used to selectively extract water from plant tissues for stable isotope analysis <sup>[3, 5]</sup>. But since CVD's first use and subsequent wide spread adoption, a variety of other methods have been developed to access plant and soil-pore water for stable isotope analysis <sup>[3, 4, 6]</sup>. Recently, there have been calls for intercomparison of these techniques <sup>[4, 7]</sup>. Work with soil water extraction methods has shown significant differences in returned stable isotope values of extracted water, based on the extraction method used <sup>[3, 8]</sup>. But to date, no formal inter-method comparison of the systems used to extract water samples from plants for isotopic analysis of  $^2\text{H}/^1\text{H}$  ( $\delta^2\text{H}$ ) and  $^{18}\text{O}/^{16}\text{O}$  ( $\delta^{18}\text{O}$ ) ratios has yet been carried out.

Here we compare six available extraction systems used to selectively extract water from plant tissues for stable isotope measurement of  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  ratios. Our null hypothesis is that all extraction methods will return the same water isotopic composition. Our work thus focuses on

extraction system accuracy, repeatability, and sample purity. The plant used in our study is spring wheat (*Triticum aestivum* L.). We compare the following extraction methods: direct vapour equilibration (DVE) <sup>[9, 10]</sup>, high pressure mechanical squeezing (HPMS) <sup>[11]</sup>; centrifugation <sup>[2]</sup>; cryogenic vacuum distillation in two forms based on: a) Orłowski *et al.* <sup>[12]</sup> (hereafter CVD-1) and b) Koeniger *et al.* <sup>[13]</sup> (hereafter CVD-2); microwave extraction off-axis integrated cavity output spectroscopy (ME-OA-ICOS) analysis method, based on Munksgaard *et al.* <sup>[14]</sup>.

Our specific questions were:

- i. How do the returned stable isotope results compare between the different systems of extraction?
- ii. Do the co-extracted volatile organic compounds methanol and ethanol affect the returned isotopic results?
- iii. Which extraction method has the best sample purity, repeatability, and throughput?

### 2.3 Materials and Methods

Spring wheat (CDC-Utmost cultivar) was grown in a 0.6 m<sup>3</sup> container with a 0.50 m potential rooting depth, outdoors in a small greenhouse in Saskatoon, Saskatchewan, Canada. The soil used for the trial (medium-fine sandy topsoil) was thoroughly mixed and characterized (Table S1) prior to being placed in the trial container. To determine the initial soil water isotopic conditions, a total of six soil samples were collected prior to seeding. Wheat was planted in four rows lengthwise down the container with 10 cm spacing between rows and 2.5 cm seed spacing in the row. The wheat was irrigated every two to three days over the course of its growth, with a minimum of 10 L of water used per irrigation period. Irrigation water samples were collected at each irrigation period and measured for  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  values (Table S2). Wheat samples were collected at 56 days post seeding near the end of the flowering stage, prior to hardening. Each collected whole plant, was split into four portions: head, stems, leaves and root crown. Our previous pilot testing with spring wheat and the CVD-1 system had shown that the various plant portions returned markedly different stable isotope results from one another. Each portion was

weighed prior to sealing them into their appropriate extraction method container. Volumes of plant matter appropriate to each method of extraction were collected. Five replicates per plant portion, per method of extraction were collected for a total of thirty samples per extraction method. During plant sampling, soil samples were collected in two locations bounding either side of the collected wheat samples. Soil samples were collected with five replicates per 10 cm depth to the bottom of the container for a total of 50 soil samples. Soil pore water was extracted using HPMS as per Orłowski *et al.* [3].

Plant samples were stored in glass vials for CVD, ME-OA-ICOS and centrifugation. For HPMS, samples were stored in 250 ml Nalgene™ HDPE bottles. Care was taken to minimize headspace in the sealed containers to limit mixing with atmospheric water vapour internal to the container over the storage period. The glass vials and Nalgene™ bottles were tightly sealed by lids, wrapped with Parafilm®, and stored in a cooler at 4 °C to minimize potential evaporation during storage, prior to extraction. The DVE samples were stored in Ziploc® double zipper freezer bags and analyzed within 48 hours of sampling. Extraction/equilibration protocols and method specific preparations are described in detail below. To determine extraction efficiency, prior to and after extraction, initial plant and soil sample weights were compared to weights taken after extraction and again after a 24-hour drying period at 105 °C. All extracted liquid samples were filtered with 0.45-µm disk filters and transferred to 2-mL glass vials, capped, and sealed with Parafilm® prior to isotopic analysis.

### *2.3.1 Plant Water Extraction Techniques and Extraction Parameters.*

*Direct Vapour Equilibration.* Plant samples were macerated prior to being placed inside of 945-mL Ziploc® double zipper freezer bags. Plant material quantities used per replicate were: 8.0 g of head material, 7.0 g of leaf material, 14.0 g of stem material, 5.0 g of root material. We followed protocols developed by Wassenaar *et al.* [10]. Bags were inflated with dry air immediately after sampling and allowed to equilibrate for 24 hours at room temperature (~22 °C) prior to analysis. Isotopic analysis of the bag headspace was made on a Los Gatos Research IWA-45EP OA-ICOS analyzer (Los Gatos Research Inc., San Jose, CA, USA) in vapour mode, which allows for continuous flow headspace sampling. In vapour mode this instrument measured

H<sub>2</sub>O concentration and the H<sub>2</sub>O isotopologues at 5 s integration intervals. The headspace sampling apparatus was a 21 G stainless steel needle connected to a 1 m long by 0.95 mm impermeable plastic line that was attached to the input port of the LGR IWA-45EP analyzer. Prior to, and in between sampling of headspace, the needle was connected to a Drierite laboratory gas drying unit (W.A. Hammond Drierite Co. Ltd, Xenia, OH, USA) until the internal water content of the IWA-45EP analyzer was below 2000 ppmV H<sub>2</sub>O. The headspace of plant and water standard bags were sampled by piercing the sample bag with the needle. Sampling took on average two minutes. Water standard bags were re-sealed with tape in between analyses.  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  values were noted when the measured headspace water content stabilized at ~28,000 ppmV H<sub>2</sub>O for at least one minute. Two lab water standards of known isotopic composition were used to correct for drift. Standard one had isotopic composition -137.5‰ ( $\delta^2\text{H}$ ) and -17.45‰ ( $\delta^{18}\text{O}$ ). Standard two had isotopic composition 2.5‰ ( $\delta^2\text{H}$ ) and -0.1‰ ( $\delta^{18}\text{O}$ ). 20 mL of the water standard was placed into a 945-mL Ziploc® bag, filled with dry air and allowed to equilibrate for 1 hour prior to analysis. Water standards were alternated and analyzed every four samples. A third, known, control water sample was analyzed every eight samples as a means of checking our drift corrections. The control water isotopic composition was, -201‰ ( $\delta^2\text{H}$ ) and -26.17‰ ( $\delta^{18}\text{O}$ ).

*Cryogenic Vacuum Distillation.* The CVD-1 system comprised six separate extraction units, each with four extraction-collection lines. This allowed for the extraction of up to 24 samples at a time. Our modification to the original CVD-1 <sup>[12]</sup> system included the addition of 1 extra extraction-collection line per extraction unit, and the removal of the nitrogen purging system. System protocols for sample extraction included heating the sample material to 100 °C for 210 minutes under baseline vacuum pressures of 0.1 Pa. The total volatile fraction in the plant samples was vapourized and collected in a liquid nitrogen cold trap. Once extractions were completed, the collected analyte was defrosted at room temperature, in sealed conditions, and the liquid was sampled for subsequent isotopic analysis. To ensure appropriate analytical volumes of extracted water, 6.0 g of head material, 6.0 g of leaf material, 11.0 g of stem material, and 5.0 g of root material were used per replicate.

The CVD-2 system used in this study was based on the design detailed in Koeniger *et al.* <sup>[13]</sup>. This system was composed of independent extraction-collection units made up of two Labco Exetainer® (Labco Ltd, Lampeter, Ceredigion, UK) vials connected by a 2.00 x 0.95 mm stainless steel capillary. Samples were heated to 200 °C for 15 min under a baseline vacuum pressure of 87.0 Pa. The volatile fraction in the plant sample was vapourized and was collected in the second exetainer, set in a liquid nitrogen cold trap. Samples were defrosted at room temperature in sealed conditions and the collected liquid was sampled for isotopic analysis. To ensure appropriate analytical volumes of extracted liquid 5.0 g of head material, 5.0 g of leaf material, 10.0 g of stem material, and 4.0 g of root material were used per replicate.

*Microwave Extraction.* For each plant portion extracted (head, leaf, stem, and root) a 5.0 g sub sample of each replicate was macerated and distributed evenly on the base of the extraction vessel. Our ME-OA-ICOS system was constructed based on Munksgaard *et al.* <sup>[14]</sup>. A Panasonic™ model NN-ST6615 (Panasonic Corporation, Kadoma, Osaka Prefecture, Japan) household microwave and extraction system was connected to a Los Gatos Research IWA-45EP OA-ICOS analyzer. The system utilized microwave irradiation at 300 W for 15 minutes (equivalent to ~60-80 °C), volatile components were evaporated from the samples in a sealed container and passed into a dry air stream. This air stream moved through a cooled condensation chamber that controlled the vapour concentration and flow rate into the analyzer. To calibrate and correct for drift, liquid water standards were run for every fourth sample. The same water standards as were used in the DVE extractions were used in the ME-OA-ICOS extractions. For this step a piece of Whatman® 541 filter paper was placed inside the extraction vessel and 0.3 ml of standard water was added to the filter paper as per Munksgaard *et al.* <sup>[14]</sup>. The same extraction procedure was used on the isotope standards. Isotope ratios for  $\delta^{18}\text{O}$  and  $\delta^2\text{H}$  values were calculated by applying machine specific corrections to the sample data set based on the protocols in Schmidt *et al.* <sup>[15]</sup>.

*Centrifugation.* We followed the centrifugation method of Peters and Yakir <sup>[2]</sup>. Centrifugation vials were a modification of the vials used in Peters and Yakir <sup>[2]</sup>. Sampled plant material was placed in 15 ml centrifugation vials. These vials were frozen with liquid nitrogen to limit



evaporation from the plant matter during a subsequent 20 second maceration process [2]. A cap with a centrally located 3.0 mm hole, with layered fine stainless-steel mesh and two pieces of Whatman® grade 1 filter paper was placed at the base of the 15 ml centrifugation vials. These vials were then inverted and placed into a larger 50 ml centrifugation vial, thus allowing liquid to move from the sample and collect in the larger centrifugation vial. After the maceration process the samples were re-warmed to room temperature under sealed conditions prior to centrifugation. All plant samples were spun at 10,000 rpm (16,000 g) for 30 minutes at 4 °C in Beckman-Coulter Avanti™ JXN-26 centrifuge (Beckman-Coulter Inc., Brea, CA, USA). Previous pilot testing had shown that for times less than 30 minutes, extraction efficiencies were low, and for times beyond 30 minutes no more liquid could be extracted from the samples. To ensure appropriate analytical volumes of extracted liquid, 9.0 g of head material, 7.0 g of leaf material, 9.0 g of stem material, and 14.0 g of root material were used per replicate. The samples collected after initial centrifugation contained high amounts of co-extracted organic compounds as noted by a dark discolouration (Figure 1). Particulate matter was removed from the analyte by a second centrifugation step in an Eppendorf 5804 centrifuge (Eppendorf, Hamburg, Germany) at 11,000 rpm (~8000g) for 1 hour. Samples were then filtered with 0.45 µm disk filters into 2 ml glass vials prior to isotopic analysis. Samples retained the noted colour after the second centrifugation and filtering.

*High Pressure Mechanical Squeezing.* Our HPMS approach followed Bottcher *et al.* [11]. The squeezers were composed of a stainless-steel chamber, a porous titanium filter disk overlaying the exit port, and a stainless-steel piston, which applied compression to the top of the sample. Both plant and soil samples that underwent HPMS extraction were placed in the chamber and squeezed at a pressure of 10,000 PSI (69.8 MPa), for 24 hours. Liquid expelled by the plants and soils, were collected via a syringe attached to the output port, filtered through a 0.45 µm syringe filter and stored in 2 ml glass vials prior to isotopic analysis. Care was taken to prevent the samples from contacting the atmosphere during the extraction process. To ensure appropriate sample volumes, 6.0 g of head material, 5.0 g of leaf material, and 10.0 g of stem material, were used per replicate. The root sample replicates did not produce enough liquid with this method of extraction and were combined into a single composite sample.

### 2.3.2 Isotope Analyses.

To rule out any potential analytical differences, when possible, the extracted liquid samples were analyzed on both laser based off-axis integrated cavity output spectroscopy (OA-ICOS), and isotope-ratio mass spectrometry (IRMS). It has been noted in previous studies that liquid samples extracted from plants often contain co-analytes such as methanol, ethanol, phenolics, acids, terpenes, sugars, proteins, and glycols<sup>[16-18]</sup>. These co-analytes can interfere with spectrometric measurements but are believed to be in low enough concentrations that they do not impact mass-based analysis systems. We note that previous work has shown that the two major isotope ratio infrared spectroscopy (IRIS) analysis systems: Picarro's cavity ring down spectroscopy (CRDS) (Picarro, Santa Clara, CA, USA) and LGR's OA-ICOS, return significantly different results when exposed to the same amounts of methanol and ethanol contamination<sup>[14, 17, 18]</sup>. The centrifugation and HPMS samples could not be analyzed on OA-ICOS due to the high content of co-extracted compounds. The highly viscous nature of the centrifuged and squeezed samples prevented proper injection into the sampling chamber of the OA-ICOS analyzer. Extracts from soil samples were analyzed only on the OA-ICOS system as they were not flagged for organic contamination during OA-ICOS analysis.

For OA-ICOS analyses, a Los Gatos Research IWA-45EP OA-ICOS analyzer was used with an accuracy of  $\pm 1.0\text{‰}$  for  $\delta^2\text{H}$  and  $\pm 0.2\text{‰}$  for  $\delta^{18}\text{O}$  values. For water vapour measurements, the ME-OA-ICOS and DVE samples were analyzed on the IWA-45EP system. In vapour mode, for a 30 second reading period, results were accurate to  $\pm 1.8$  for  $\delta^2\text{H}$  and  $\pm 0.3$  for  $\delta^{18}\text{O}$  values. The isotopic data for soil and plant extracts from the OA-ICOS system were checked for spectral contamination using the Spectral Contamination Identifier (LWIA-SCI) post-processing software (Los Gatos Research Inc.) All plant samples extracted with the CVD-1 and 2 systems, analyzed on the IWA-45EP, were flagged for narrow band and broad band spectral contamination, noting the presence of organic contaminants.

For IRMS analyses, an Elementar Isoprime isotope ratio mass spectrometer (Elementar UK Ltd, Stockport, UK) was used. The hydrogen isotopic compositions were determined by reduction of water to hydrogen by reaction with elemental chromium, following the method of Morrison *et al.*

[19]. System protocols were as follows: 0.8  $\mu\text{L}$  of water was injected into a quartz reactor containing elemental chromium at 1030  $^{\circ}\text{C}$ . The resultant  $\text{H}_2$  gas was separated on a 1 mmole sieve gas chromatography column and introduced into the Elementar Isoprime isotope ratio mass spectrometer. For the CVD-1 samples, memory effects were reduced by injecting two replicates of each sample and discarding the first measurement. The CVD-2, Centrifuge and HPMS samples were injected between two and six times, due to the more viscous nature of the liquid and the high content of co-extracted organic content. In this case, we then discarded all but the last two injections results. Resultant raw delta values of the measured hydrogen were normalized to the VSMOW-SLAP [20] scale by analyses of two calibrated waters, INV1 and ROD3, with  $\delta^2\text{H}$  values of -218 and -4 ‰, respectively. For oxygen isotopes, we used the  $\text{CO}_2\text{-H}_2\text{O}$  equilibration technique of Epstein and Mayeda [21]. A Micromass multi-flow device (Waters Corp., Milford, MA, USA) was connected to the Elementar Isoprime isotope ratio mass spectrometer at 25  $^{\circ}\text{C}$  for all  $\text{CO}_2\text{-H}_2\text{O}$  equilibrations. Results are reported relative to the VSMOW-SLAP scale by normalizing to INV1 and ROD3, with  $\delta^{18}\text{O} = -28.5\text{‰}$  and  $-1.0\text{‰}$ , respectively, as above. The mass spectrometer used in this study had a precision of  $\pm 1.5\text{‰}$  for  $\delta^2\text{H}$  and  $\pm 0.3\text{‰}$  for  $\delta^{18}\text{O}$  values.

The isotope ratios (R) for  $\delta^2\text{H}$  ( $^2\text{H}/^1\text{H}$ ) and  $\delta^{18}\text{O}$  ( $^{18}\text{O}/^{16}\text{O}$ ) are expressed in per mil (‰) relative to Vienna Standard Mean Ocean Water (VSMOW), defined as follows:

$$\delta^2\text{H or } \delta^{18}\text{O} = (\text{R}_{\text{SAMPLE}} / \text{R}_{\text{STANDARD}} - 1) \quad (1)$$

Where  $\text{R}_{\text{SAMPLE}}$  is the  $^2\text{H}/^1\text{H}$  and  $^{18}\text{O}/^{16}\text{O}$  ratios of the sample.  $\text{R}_{\text{STANDARD}}$  is the  $^2\text{H}/^1\text{H}$  and  $^{18}\text{O}/^{16}\text{O}$  ratios of the VSMOW standard [20, 22].

### 2.3.3 Quantifying Methanol and Ethanol Content.

As there is currently no appropriate method, such as the z-scores used by Wassenaar *et al.* [23], for gauging extraction system accuracy when applied to plant water extractions, we discuss accuracy in terms of the purity of water extracted by that system. The inability to utilize a z-score type method comes from the difficulty in defining a reference water value in studies of this type. Since co-extracted organic compounds are known to cause errors in stable isotope results when

extracted liquids are analyzed by spectrometric methods (CRDS/OA-ICOS) <sup>[17, 18]</sup>, any extraction system that produces a low purity analyte has the potential to be less accurate than those that produce extracted water of a higher purity. Liquid samples from CVD-1, CVD-2, HPMS and centrifugation systems were analyzed for methanol and ethanol content, as these were the two most often noted co-extracted compounds causing spectral contamination during analysis on laser-based (CRDS and OA-ICOS) spectrometers<sup>[17, 18]</sup>. During analysis of DVE and ME-OA-ICOS samples, we closely monitored the absorption plots produced by the LGR IWA-45EP OA-ICOS analyzers. This plot, produced in real time during analysis, will show fluctuations between absorption peaks when spectral contaminants are present in the analysis stream. A further tool for assessing the presence of spectral contaminants during analysis on LGR systems is the ‘Spectra Fit Residuals plot’ (SFR), which has higher sensitivity to the presence of contaminants than the absorption plots. The SFR plot was not available for use during this study. The Saskatchewan Research Council-Environmental Analytics Laboratory analyzed liquid water samples, via gas chromatography-flame ionization detector (GC-FID) method for methanol and ethanol content. Results are reported in µg/ml.

#### *2.3.4 Statistical Analyses.*

Analysis was carried out with the statistical software R 3.3.2.<sup>[24]</sup> To quantify consistency and repeatability, mean and standard deviation (sd) were calculated for the replicates of the various plant portions across all methods of extraction, and for both systems of analysis. Standard dual isotope plots [ $\delta^{18}\text{O}$ ,  $\delta^2\text{H}$ ] were used to discern general trends.

Plant and soil water stable isotope data were tested for normality using histograms, quantile-quantile plots, and the Shapiro-Wilk normality test<sup>[25, 26]</sup>. The plant water data, grouped by extraction method, was found to be normally and non-normally distributed. Thus a non-parametric Kruskal-Wallis (one-way ANOVA on ranks) test<sup>[27, 28]</sup> was used to determine if the population medians, separated by plant portion extracted (head, leaf, stem, and root crown) were statistically similar across the extraction methods used in this study. Subsequently, Dunn’s test<sup>[29]</sup> was used to determine which of the extraction methods produced significantly different results for the plant portion in question ( $p \leq 0.05$ ). To control false discovery rates and adjust p-

values, the Benjamini-Hochberg adjustment was used<sup>[30]</sup>. The soil water data was found to be normally distributed by Shapiro-Wilk test. However, since normality tests don't have the power to reject  $H_0$  for small sample sizes, these small samples will often pass normality tests<sup>[26]</sup>. Q-Q normal plots of the soil data implied non-normality. Thus the non-parametric Mann-Whitney-Wilcoxon test<sup>[31, 32]</sup> was used to determine which of the soil layers were significantly different from one another at a 95% confidence interval ( $p \leq 0.05$ ).

## 2.4 Results

Table 1 summarizes the descriptive statistics for the extracted isotopic ratios of all plant portions, for all methods of extraction, and for each method of isotopic analysis (OA-ICOS and IRMS).

The stable isotope signatures of irrigation water used in this study were -128.25‰ (sd: 0.65,  $n=18$ ) for  $\delta^2H$  and -15.80‰ (sd: 0.11,  $n=18$ ) for  $\delta^{18}O$ . For  $\delta^2H$  results, the CVD-1 and CVD-2 systems were the most repeatable across the various plant portions, except for the stem results for CVD-2. DVE, centrifugation, and HPMS methods were moderately reproducible, decreasing in reproducibility respectively. The ME-OA-ICOS system was the least reproducible with the highest and most consistently unacceptable standard deviations. For  $\delta^{18}O$  results, CVD-1 had the most reproducible results, followed by centrifugation, HPMS, DVE, CVD-2 and ME-OA-ICOS. Across all plant portions, CVD-1 had a standard deviation range of 0.86 to 2.87 for  $\delta^2H$  and 0.34 to 1.22 for  $\delta^{18}O$ . CVD-2 had a standard deviation range of 1.50 to 15.70 for  $\delta^2H$ , and 1.52 to 8.75 for  $\delta^{18}O$ . DVE had standard ranges of 1.48 to 6.92 for  $\delta^2H$  and 0.64 to 3.41 for  $\delta^{18}O$ .

Centrifugation sd ranges were 0.88 to 6.78 and 0.30 to 1.45 for  $\delta^2H$  and  $\delta^{18}O$  respectively.

HPMS had sd ranges of 4.23 to 6.92, and 0.58 to 0.95 for  $\delta^2H$  and  $\delta^{18}O$  respectively. Finally,

ME-OA-ICOS had standard deviation ranges of 5.50 to 13.70 for  $\delta^2H$  and 1.27 to 2.91 for  $\delta^{18}O$ .

Figure 2 summarizes the stable isotope results for the extracted soil profile water. Analysis by Mann-Whitney-Wilcoxon test showed that the soil was split into significantly different horizons. The  $\delta^2H$  data shows the 0-10 cm, 10-40 cm, and 40-50 cm layers to be significantly different from one another. The  $\delta^{18}O$  data shows the 0-10 cm, and 10-50 cm, layers to be significantly different from one another. Stable isotope signals in all layers were more enriched in heavier

isotopes than the irrigation water and were progressively enriched in the heavier isotopes towards the soil surface with the 0-10 cm layers being most enriched for both  $^2\text{H}$  and  $^{18}\text{O}$ .

#### *2.4.1 Plant Portion and Extraction Method Differences.*

The plant portions sampled followed a similar pattern of enrichment/depletion of measured  $\delta$ -values through all systems of extraction. Figures 3 and 4 show the irrigation, plant, and soil water results in standard dual isotope space. For the plant water samples, the stem results were the most depleted in heavier isotopes ( $^2\text{H}$ ,  $^{18}\text{O}$ ) over all extraction systems. The root crown results were more enriched in  $^2\text{H}$  and  $^{18}\text{O}$  than the stems and tended to closely resemble the 0-10 cm soil layer  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  values, except for the CVD-1 system root crown results, which more closely resembled the 20-50 cm soil layers. The leaf and head plant water results were the most enriched in  $^2\text{H}$  and  $^{18}\text{O}$  for all extraction systems. The leaf and head results were similar to each other for all extraction systems, except in the case of the HPMS and centrifuge results. For HPMS, the heads were more enriched in  $^2\text{H}$  and  $^{18}\text{O}$  than the leaves. For centrifugation the opposite was true, with the leaf water results being more enriched in  $^2\text{H}$  and  $^{18}\text{O}$  than the heads.

#### *2.4.2 Co-extracted Organics and Resultant Differences Between OA-ICOS and IRMS Analyses.*

Only CVD-1 and 2 methods produced a liquid analyte that could be run on the two methods of liquid analysis: OA-ICOS and IRMS. The other methods of extraction either did not produce liquid, as in the case of DVE and ME-OA-ICOS, or produced a liquid that was too viscous, due to co-extracted compounds, to be run on the OA-ICOS system. Methanol and ethanol content of water from all extraction systems that provided liquid analyte, are summarized by plant portion in Table 2. For co-extracted ethanol content, HPMS and centrifugation produced the largest concentrations, followed by CVD-2 and then CVD-1. For co-extracted methanol content, HPMS and centrifugation again produced the largest concentrations, followed by CVD-2. CVD-1 produced one to two orders of magnitude less methanol than the other systems.

For results from the CVD-1 extraction system, stable isotope differences between methods of analysis (IRMS vs OA-ICOS) for all plant portions ranged from 0.03‰ to 5.10‰ (average: 1.54‰, sd: 1.23) for  $\delta^2\text{H}$  and, 0.21‰ to 1.66‰ (average: 0.78‰, sd: 0.44) for  $\delta^{18}\text{O}$ . For the

CVD-1 system, methanol and ethanol content was highest in the heads, followed by stems, leaves, and then root crowns. While the CVD-1 system results were flagged for narrow and broad band contamination on the OA-ICOS systems, the presence of co-extracted methanol and ethanol did not appear to be large enough to significantly skew the results between the two analysis systems (IRMS vs OA-ICOS). Though the boiling point of methanol and ethanol are 64.7 °C and 78.2 °C respectively, the extraction temperature of 100 °C used by the CVD-1 system did not appear to co-extract enough methanol/ethanol to negatively affect the results of OA-ICOS analysis for spring wheat. For the CVD-2 extraction system, differences between methods of analysis (IRMS vs OA-ICOS) for all plant portions ranged from 0.61‰ to 28.61‰ (average: 6.26‰, sd: 5.95) for  $\delta^2\text{H}$ , and 0.24‰ to 20.73‰ (average: 6.13‰, sd: 5.62) for  $\delta^{18}\text{O}$ . The methanol and ethanol content extracted by the CVD-2 system was nearly an order of magnitude higher for all plant portions than that extracted by the CVD-1 system, and this is likely due to the higher extraction temperature of 200 °C. For the CVD-2 system, ethanol content was highest in the heads, followed by stems, leaves, and then root crowns. However, methanol content was highest in stems, followed by heads, leaves and root crowns. The results of the CVD-2 system were also flagged for narrow and broad band contamination on the OA-ICOS analysis system and, we believe that the larger differences between the CVD-2 IRMS and OA-ICOS results are likely a byproduct of these co-extracted volatile organic compounds (VOCs). Centrifugation and HPMS co-extracted methanol and ethanol in quantities one to two orders of magnitude higher than the CVD-1 method. Also, depending on plant portion, the centrifuge and HPMS systems typically produced more methanol and ethanol than the CVD-2 system. We expected minimal amounts of methanol and ethanol to be present in the headspace of DVE samples as the DVE extraction-analysis step takes place at room temperature (~22 °C). At this temperature, based on equilibrium thermodynamics of partial pressures, minimal volumes of methanol and ethanol would be volatilized during the equilibration of plant water with the dry headspace of the extraction vessel. To this point, the DVE samples did not show evidence of spectral contamination on the LGR IWA-45EP OA-ICOS analyzer's absorption plot during analysis. However, this is not conclusive evidence for the lack of contaminants in the headspace of plant samples. Further research is needed to quantify volumes of organic contaminants potentially present in the headspace of DVE samples.

### 2.4.3 Variance Analysis.

The Kruskal-Wallis one-way ANOVA by ranks test demonstrated significant differences in the isotopic results between the methods of extraction for each plant portion. The results of this test are summarized in Figure 5. Overall, for all plant portions (PP) and isotopes, the ME-OA-ICOS system had the greatest number of significantly different results, followed by CVD-1, HPMS, centrifuge, DVE and finally the CVD-2 system. Generally, for  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  values, the CVD systems produced results which were similar to each other for all PP. The CVD-2 system (OA-ICOS and IRMS results) produced the least number of significantly different results across all PP, but the CVD-2 OA-ICOS results had fewer significant differences than the CVD-2 IRMS results for all PP. Again, we note the relatively higher amount of co-extracted VOCs in the CVD-2 samples, which may account for the differences between OA-ICOS and IRMS results. The CVD-1 OA-ICOS and IRMS results had more significant differences for  $\delta^2\text{H}$  than  $\delta^{18}\text{O}$  results across all PP and were inconsistently different depending on the plant portion of interest. The mechanical methods HPMS and centrifugation produced results which were not significantly different for  $\delta^2\text{H}$  values. However, for leaf and stem portions, the  $\delta^{18}\text{O}$  results were significantly different between these two methods. HPMS results had far more significant differences for  $\delta^{18}\text{O}$  than  $\delta^2\text{H}$  values. The opposite was true for centrifugation which provided a greater number of significantly different results for  $\delta^2\text{H}$  than  $\delta^{18}\text{O}$  values. Even though the ME-OA-ICOS and CVD systems are based on similar mechanisms, the ME-OA-ICOS results tended to be consistently different from the CVD-1 and CVD-2 results. The ME-OA-ICOS system produced more significantly different results for  $\delta^2\text{H}$  than  $\delta^{18}\text{O}$  values. Overall, the DVE method produced the second lowest number of significantly different results. DVE had a similar number of significant differences for both  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  results and it produced results that were inconsistently different for both isotopes depending on the plant portion sampled. When DVE was compared to the standard method, cryogenic vacuum distillation, DVE produced results that were not significantly different from the CVD-2 system's results for all PP, except for  $\delta^2\text{H}$  results from leaf tissue. However, consistent differences between DVE and CVD-1  $\delta^2\text{H}$  values are noted, but the  $\delta^{18}\text{O}$  results were consistently similar between these methods.



#### *2.4.4 Extraction Efficiencies and Sample Throughput*

The CVD-1 and CVD-2 systems had extraction efficiencies of 99.8% (sd: 1.26) and 98.1% (sd: 0.34) respectively. The ME-OA-ICOS had an average extraction efficiency of 98.2% (sd: 2.27). Thus, for methods utilizing heat to extract analyte from samples, we are confident that no Rayleigh fractionation processes would have affected our results. The HPMS system had an average extraction efficiency of 82.1% (sd: 9.24). Increasing pressure beyond 69.8 MPa was not possible with our system and extracting samples for longer than the 24 hours noted in the HPMS protocol did not increase the extraction efficiency. Centrifugation had the lowest extraction efficiencies at 48.9% (sd: 9.60) on average. We found that increasing the rotational speed and duration beyond 10,000 rpm (16,000 g) and 30 minutes respectively did not further improve extraction efficiencies.

Sample throughput rates for the extraction/analysis methods are calculated per 8-hour period. DVE had the highest sample throughput with 74 samples followed by CVD-2 system with 60 samples. We note that the CVD-2 throughput rate is a product of heating block specifications and could be improved with a larger heating apparatus. Centrifugation had a throughput of 38 samples. The CVD-1 system had a throughput of 24 samples, which is dependent upon the number of extraction-collection lines added into the system. We note that the addition of extra extraction-collection lines to this system results in diminishing returns on sample throughput, as additions increase the setup and post-extraction sampling time. The ME-OA-ICOS system requires a cool-down period after each sample resulting in a throughput of 16 samples. The HPMS system had the lowest sample throughput which is a byproduct of requiring 24 hours to extract one sample. Sample throughputs with the HPMS system can be increased by adding more squeezing units. We had access to three squeezing units for this study and could process three samples every 24 hours. At the end of the extraction period described here, only the DVE system immediately returns isotopic values. The cryogenic systems, HPMS and centrifugation provide a liquid that will then require another 24-48 hours of analysis on either IRMS or OA-ICOS analyzers before isotopic values are available. Both IRMS and OA-ICOS analyzers also have sample throughput rates which limits speed of returned data. The ME-OA-ICOS system also

provides delta values and isotopic ratios during analysis. However, the raw ME-OA-ICOS data must first go through a post processing integration and correction step, before it is useful.

## **2.5 Discussion:**

### *2.5.1 Methodological Controls on Accessible Water Pools Within the Plant.*

The methods of extraction used in this study can be split into two groups, defined by the way they extract water from a sample: (1) ‘mechanical methods’ like HPMS and centrifugation that use physical force to push liquid out of the samples and (2) ‘phase change methods’ like CVD-1, CVD-2, ME-OA-ICOS and DVE. Cryogenic and microwave methods rely on heat and pressure changes to volatize the liquid in the sample and pull it out of the sample for collection/analysis. The DVE system is unique in that while it relies on the phase change of water, no heat or pressure changes are used to induce this phase change, only equilibrium processes.

It is also possible to group the methods of extraction by the water pools and the hydrogen and oxygen pools within the plant that they are capable of accessing. Here we define the total water pool as all available water within a plant sample, that is: the relatively more mobile xylem and inter-cellular water; and the relatively less mobile intra-cellular, cell wall, and organelle constrained water. The mobile, transpiration water, is defined as that which is taken up by roots and rapidly moved through the xylem to sites of transpiration. Depending upon a water molecule’s path through the plant, (diffusional vs bulk flow), it will have greatly different residence times. This is important as we know that precipitation isotope signals and available soil water isotope signals change seasonally. Thus, a mixture of stable isotope signals connected to different uptake periods in time will be present simultaneously in a plant’s total water isotope signal. For studies interested in investigating the sourcing of water by plants, it is critical to be able to connect plant water isotope signals with the slice of time that plant sampling occurred in.

For hydrogen and oxygen pools within plants, it has been previously noted that isotopic fractionation of H and O occurs during primary production<sup>[33]</sup>. In the case of hydrogen, plants have been shown to preferentially use <sup>1</sup>H in the production of metabolic and other organic compounds. For oxygen, it is the isotopic composition of the available plant water at the time of

its production that determines the  $\delta^{18}\text{O}$  signature of organically bonded cellulose<sup>[33]</sup>. These isotopic signals are thus laid down in cellulose and other plant organic compounds during their production and may be accessible by virtue of the way water is extracted by our tested extraction systems.

The CVD-2, CVD-1 and ME-OA-ICOS methods, extract up to 99% of the water in a sample, and are thus accessing total plant water. This may serve to produce results that are more depleted in  $^2\text{H}$  and  $^{18}\text{O}$ , due to extraction of the water and H/O pools that are more enriched in  $^1\text{H}$  (depleted in  $^2\text{H}$ ). Indeed, our results for both CVD-1 and CVD-2 extraction systems were depleted in  $^2\text{H}$  and  $^{18}\text{O}$  relative to the all systems average. HPMS and centrifugation are also capable of accessing the total plant water pool, however they do so through destruction of tissue, bursting cell walls and organelles and allowing the water contained therein to be extracted along with the more mobile xylem and inter-cellular water. However, it is this destruction of tissue that means the HPMS and centrifugation systems are also accessing organic compound bound hydrogen and oxygen pools. These organic compound pools may not be the same as those extracted by the CVD and ME-OA-ICOS systems, and thus their effect on  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  values may also be different. The high content of co-extracted compounds produced by HPMS and centrifugation are observed in Figure 1 and Table 2.

#### *2.5.2 On the Accuracy of Extraction Methods Based on Co-extracted Organic Compound Content.*

While we are primarily interested in the transpiration water within plants, co-extraction of organic compounds can occur simultaneous to water extraction, the amount of which appears to depend on the extraction method used. These co-extracted organic compounds such as methanol, ethanol, phenolics, terpenes and other xylem sap constituents such as sugars, and proteins can influence the returned stable isotope signals during analysis <sup>[3, 16, 34, 35]</sup>. We quantified only the methanol and ethanol content present in extracted liquid analyte. Typically, extracted plant analyte is analyzed via IRMS, as the presence of co-extracted contaminants are often in small enough volumes relative to the total mass of water H and O, that they will not impact the accuracy of results. The rise of low cost, rapid analysis by refractive laser based spectroscopy

like CRDS and OA-ICOS, has led a number of groups to attempt to utilize this analysis method on plant water<sup>[16]</sup>. Unfortunately, the problematic co-extracted compounds have optical absorption characteristics similar to water and can thus result in errors in the measurement of  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  values on laser-based (CRDS and OA-ICOS) spectrometers<sup>[3, 4, 16]</sup>. Some extraction systems may, as a by-product of the methods of extraction, produce greater amounts of co-extracted compounds, resulting in analyte of lower purity. However, little work has been done to quantify how much and what type of co-extracted compound is produced by the various extraction systems. The various co-extracted compounds may each be contributing different  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  signals to the total water signal, based on the H and O originally used at the time of their synthesis. These various signals are currently difficult to disentangle from the total water isotope signal and may require modelling to fully understand.

Table 2 shows that the mechanical methods co-extracted methanol and ethanol in quantities one to two orders of magnitude greater than the CVD-1 method did. In addition to the visually observed contamination shown in Figure 1, HPMS and centrifugation produced a liquid that was far more viscous than that produced by the CVD-1 and CVD-2 methods. The co-extraction of organic compounds by centrifugation and HPMS makes us consider the final extracted analyte as more of a jelly, containing water and organic contamination at magnitudes large enough that our returned  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  results from these systems may be unrepresentative of the water contained within the plant, even though they were analyzed via IRMS. We therefore conclude that the HPMS and centrifugation methods are producing less accurate results due to the relatively high content of co-extracted organic compounds and the low purity of their returned analyte.

Interestingly, the CVD-2 returned stable isotope results were not significantly different from those of the DVE system for all plant portions and isotope ratios of interest, except for the CVD-2-IRMS leaf water  $\delta^2\text{H}$  results, even though the CVD-2 leaf water contained substantial amounts of methanol and ethanol. As such, the CVD-2 system may also be an acceptable extraction system for plant water analysis. However, we would recommend that a lower extraction temperature of 100 °C be used, and that analyte extracted from plant samples with this method only be analyzed via mass-based spectrometry like IRMS. The similarity between DVE and CVD-2 results could be a byproduct of the low number of samples ( $n=5$  per plant portion per method compared)

analyzed with the non-parametric ANOVA. Further research with higher N per method of extraction should be undertaken.

The DVE system is the only method that is accessing the mobile xylem and inter-cellular water pools with limited co-extraction of organic compounds. This is because DVE relies upon evaporative equilibration of water within the plant sample to that of the dry air added to the sampling container as an equilibration medium. Water contained in the xylem and within inter-cellular spaces is more mobile than water contained in the intra-cellular spaces and that of the H and O atoms contained within cellulose structures. This xylem and inter-cellular water will therefore more rapidly equilibrate with the air injected into the DVE sampling container. The more tightly held water must first diffuse through cell walls and will be slower to equilibrate with the dry air in the DVE analysis bag. We believe that the bulk of the water signal we are seeing in DVE results is that of the more mobile water pool within the plants and is therefore more representative of the water being taken up and transpired by the plant on a daily basis. For studies looking at the ecohydrological dynamics of plant water uptake, DVE could be the system that is most closely accessing the water of interest in these studies. We note lack of fluctuations between peaks in the absorption plots of analyzed DVE samples to indicate low to no presence of organic contaminants in the headspace of those samples. However, as we did not quantify the presence of organic contaminants in the headspace of the DVE samples and instead relied on observation of the absorption plot for flagging of contamination, we cannot completely rule out organic contamination issues with the DVE results.

### *2.5.3 Extraction Methods and their Effects on $\delta^{18}O$ and $\delta^2H$ Values*

The CVD-1 system, across all plant portions consistently produced the results most depleted in  $^2H$  and had the lowest standard deviation between replicates across all plant portions. The CVD-1 system is accessing total plant water by extracting ~99.8% of the water from a sample. It is likely that the returned isotopic ratios, relatively depleted in  $^2H$  are a result of this systems access to the light hydrogen ( $^1H$ ) that has been preferentially taken up into plant organic compounds. This light hydrogen pool will likely make up a greater proportion of the total water extracted, relative to the light hydrogen pools accessible to a system such as DVE. Similarly, the CVD-2

system also produced consistently depleted results for  $^2\text{H}$  for all plant portions. Again, we attribute the relatively depleted  $^2\text{H}$  results to these systems access of the plant organic compound pools. Of note, the CVD-2-IRMS  $\delta^{18}\text{O}$  results for the roots and heads had a much wider spread than its  $\delta^2\text{H}$  results, and than the CVD-2-OA-ICOS  $\delta^{18}\text{O}$  results. We attribute the CVD-2 systems relatively enriched  $^2\text{H}$  results (as compared to CVD-1 results), to the higher amounts of co-extracted methanol, associated with higher extraction temperatures. The ME-OA-ICOS system consistently produced the most enriched results for both isotope ratios across all plant portions. The spread of the ME-OA-ICOS results were also consistently the largest amongst all extraction systems, although this was more pronounced for  $\delta^2\text{H}$  results than  $\delta^{18}\text{O}$  results. Interestingly, the relatively enriched  $^2\text{H}$  and  $^{18}\text{O}$  results produced by the ME-OA-ICOS in our study are consistent with the findings of Orłowski *et al.* [3], implying that a problem with the method itself exists, wherein an operational effect results in an enrichment of the isotopic signals. We note there was difficulty during the ME-OA-ICOS extractions in maintaining seals on the extraction vessel. Samples were re-run in cases where we noted damage to the extraction vessel seals, but these leaky seals may be another source of analytical error, and enrichment of  $^2\text{H}$  and  $^{18}\text{O}$  for the ME-OA-ICOS system. The HPMS system, across all plant portions produced consistently depleted results for  $^{18}\text{O}$ , but its  $\delta^2\text{H}$  results were generally close to the average of all systems result. The centrifugation results were the least consistent of all extraction systems in terms of their depletion or enrichment of  $^2\text{H}$  and  $^{18}\text{O}$  relative to the other system's results. For the heads, centrifugation results for both isotope ratios were depleted in the heavier isotopes, but for the leaves, stems and roots, the centrifugation results tended to be enriched in  $^2\text{H}$  and relatively similar to the other extraction systems for  $\delta^{18}\text{O}$  values. The spread of the  $\delta^2\text{H}$  results for centrifugation was among the worst, while the spread of the  $\delta^{18}\text{O}$  results was consistently amongst the best. We note that due to the relatively abundant content of co-extracted organic contaminants in the mechanically extracted (HPMS and centrifugation) water samples, it may be difficult to adequately explain our isotopic results as we have not determined the full extent of what organic contaminants are present, in what quantities, and what their  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  signals are. The DVE system produced results that were more enriched in  $^2\text{H}$  and  $^{18}\text{O}$  than those of the standard cryogenic extraction systems (CVD-1 and CVD-2), we believe that this is because DVE is accessing the rapidly mobilized xylem water pools in the plant and less so the intra-cellular

and organic compound bonded H and O pools, meaning that we should expect to see more enriched  $^2\text{H}$  and  $^{18}\text{O}$  results. The spread of the DVE results were moderate relative to the other extraction systems with the  $\delta^2\text{H}$  results having more acceptable standard deviations than those of the  $\delta^{18}\text{O}$  results.

Generally our results are comparable in principle to the soil-based work of Orlowski *et al.* [3] for the extraction systems apparent effect on  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  values. The Orlowski *et al.* [3] intercomparison evaluated the methods of extraction: CVD-1, HPMS, DVE, ME-OA-ICOS, and centrifugation. For the Orlowski *et al.* [3] study, the CVD-1 system tended to produce depleted results relative to the spike water, the ME-OA-ICOS, and centrifuge systems produced results which were enriched relative to the spike signal, and DVE produced enriched results for  $^{18}\text{O}$  only and closely matched the spike signal for  $\delta^2\text{H}$  values. However, a noted difference between our results and those in Orlowski *et al.* [3] is that the HPMS system tended to produce results depleted in the heavier isotopes in our trial, whereas the HPMS system produced results enriched in  $^2\text{H}$  and  $^{18}\text{O}$  in Orlowski *et al.* [3]. In regard to significant differences between methods of extraction, their study found far more consistently different results across all systems of extraction, especially at lower soil water contents, as compared to the less consistent number of significant differences found in our study. Care should be taken when attempting to compare the result of our study with the Orlowski *et al.* [3] intercomparison, as the water carrying media (soil vs plant matter) are highly different from one another with regard to relative inertness, internal chemistry, effects on water isotope ratios and presence of co-extractable compounds.

#### 2.5.4 Extraction Method Intercomparison

While a variety of extraction methods are available for plant and soil water extraction, little work has been done to intercompare the extraction methods and their effects on the isotopic signal of the recovered water. Typically, previous method comparison studies would test a new method against one or two other extraction methods to validate the new method. [2, 14, 36-39]. The newly developed extraction method would commonly be compared to and validated against the previously established ‘gold standard’ of cryogenic vacuum distillation [5]. Orlowski *et al.* [3] carried out the first formal inter-comparison of the major lab-based soil water extraction

techniques. Their study involved the extraction of spiked soil water from two unique soil types at three water contents per soil type. They found that extraction methods returned results which were significantly different from one another depending upon soil type and water content. In fact, this work called into question the use of cryogenic extraction as the ‘gold standard’, especially when extracting water from soils with low water contents or with a highly clayey composition [3, 4, 8]. The CVD system was called into question as it produced water, whose  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  values were significantly different from the water produced by all other tested methods. The CVD  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  results were also significantly different from the spiked reference water  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  values ( $p \leq 0.05$ ) [3]. A methodological comparison of plant water extraction techniques for stable isotope analysis was carried out by Yang *et al.* [6]. They review available methods of plant water extraction, but do not investigate method performance through experiment. Ours is the first study to experimentally inter-compare the available method of plant water extraction.

The results of the ANOVA analysis above show that there are significant differences in the stable isotope ratios returned by the tested methods of extraction, depending upon the plant portion and isotope ratio of interest. However, the significant differences between extraction system’s returned values were not consistent across all plant portions tested nor for both  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  values. This implies that a variable beyond just the methodological effects of extraction may be responsible for causing significant differences between systems. We posit that the complex and varied chemistry of the different plant portions will allow for co-extraction of a unique set of compounds, depending upon plant portion sampled, that could in turn be modifying the returned stable isotope signals upon analysis on IRMS and OA-ICOS, and contributing in part to the differences between the methods of extraction. Thus, the fact that DVE may not have issues with co-extracted compounds and is primarily accessing the more mobile plant water pools further cements its usefulness as an analytical method.

#### *2.5.5 Limitations of our Study Approach and Recommendations.*

Our study is the first that we are aware of to intercompare the methods of plant water extraction for potential differences in their returned stable isotope results. However, there are limitations to



extending these results to other plant water extractions. While wheat is perhaps relatively similar to other herbaceous grass species or other soft tissue crops like maize and rice, woody biomass water extractions may be more problematic with regards to co-extractable compounds. The relative volumes of co-extracted organic compounds present in the water pools of herbaceous grasses may be vastly different from the volumetric content of VOCs in larger tree species. This could modify the result of studies intercomparing the methods of extraction on woody plant species. Further work is urgently needed to quantify types of co-extracted organics in woody plant material and their effects on the  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  signatures in returned isotopic results.

Special care should be taken when investigating inter-method differences for plant species that have historically proven problematic when run on laser-based spectrometers due to their production of high volumes of co-extracted compounds. Further to this point, future work should investigate how these co-extracted compounds affect returned  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  signals when extracted water is analyzed via OA-ICOS. Quantification of the volumes and isotopic signatures of co-extracted compounds that will cause inaccurate results; and further development of filtration techniques and post analysis correction techniques should also be undertaken. Since we advocate that the DVE system is the most appropriate method for characterizing xylem and thus source water signals in plant water sourcing studies, more work is also needed to investigate the standard operating procedures of the DVE system. Specifically, studies should be carried out investigating: the effects of various equilibration times on returned isotopic signals, and the types of dry air that are most appropriate for generating an initial isotopically un-biased non-equilibrium condition in analysis containers. Based on intended storage length, users of the DVE system should choose the most appropriate containers for storage and analysis. Hendry *et al.* <sup>[9]</sup> suggest that for storage under 10 days the Ziploc® double zipper freezer bag is acceptable, but for storage periods >30 days Sprenger *et al.* <sup>[4]</sup> recommend laminated coffee bags.

## 2.6 Conclusions:

We compared six different extraction/analysis systems used in determining the stable isotope composition of plant water, on four isotopically unique plant portions of spring wheat. Where possible we analyzed the resultant extracted liquid analyte on IRMS and OA-ICOS system to

determine if the presence of co-extracted organic compounds modified the returned isotopic results. We reject our null hypothesis that all extraction methods will return the same water isotopic composition. We found significant differences between returned stable isotope results produced by the extraction/analysis methods, depending upon the plant portion analyzed.

Inter-comparisons between the methods of analysis (IRMS and OA-ICOS) showed that for lower extraction temperatures of 100 °C, organic contamination was not significant, but that for higher extraction temperatures of 200 °C, organic contamination appeared to have a greater effect on results likely due to increasing methanol and ethanol co-extraction. Further to this, the mechanical methods of centrifugation and HPMS produced such large quantities of co-extracted compounds in their extracted analyte, that the analyte was physically unable to be analyzed on the OA-ICOS system. Thus, the analyte produced by HPMS and centrifugation may not be reasonable to compare against the other extraction method's analyte, due to the large volumes of undefined co-extracted compounds present. We recommend use of lower extraction temperatures over longer extraction periods for cryogenic methods, to limit issues with organic contamination as well as to limit potential burning or oxidation of plant tissues which may contribute to inaccurate stable isotope results.

While difficulty exists in defining a reference water value to use in quantifying the accuracy/precision in plant water extraction, we determined via investigation of the mechanisms of extraction, and by quantifying co-extracted methanol and ethanol content in other extraction methods, that DVE is likely the most appropriate method to use when investigating plant water sourcing, at least for wheat. The rapid sample throughput, portability, and near instant analytical results of the DVE-OA-ICOS system will allow for much greater temporal resolution in studies utilizing plant water isotopes. However, more research is needed to confirm DVE's utility and accuracy for other plant species.

## **2.7 Acknowledgments**

Thanks are due to Kim Janzen (for isotopic analysis), Natalie Orlowski, and the McDonnell Watershed Hydrology lab group. Also, the co-operation and collaboration of Geoff Kohler and

Environment Canada were invaluable. This research was supported by an NSERC Discovery grant to JJM, and by the NSERC CREATE program.

## **2.8 Transition Statement**

Chapter 2 has shown that there are significant differences between the tested methods of plant water extraction-analysis. Significant differences between CVD-2 and DVE were not present and this was an unexpected finding due to the high presence of co-extracted organic compounds methanol and ethanol in the water produced by CVD-2. Further work investigating the differences between CVD-2 and DVE will be carried out with a higher number of samples to compare in Chapter 3. Significant differences between the CVD-2 OA-ICOS and IRMS analysis results indicate that the presence of methanol, ethanol and other co-extracted organic compounds impacts returned stable isotope results when an IRIS method is applied for analysis. These findings set the stage for Chapter 3 (further investigation of potential method differences between DVE and CVD-2) and Chapter 4 (quantifying the effect of methanol and ethanol contamination on results returned by an LGR OA-ICOS analyser).

## **2.9 Author Contributions**

C. Millar designed the study; planted, maintained, and collected the wheat samples; carried out extraction/analysis of most plant and soil water (analyte) samples; conducted all data analysis; and wrote the manuscript. D. Pratt provided consultation on experiment design, assisted in samples collection, provided consultation on analyte extraction/analysis, and edited the manuscript. D. Schneider and J.J. McDonnell edited the manuscript and provided critical feedback and insights into understanding results, as well assisting in development of the discussion section of the manuscript.

## **2.10 References:**

- [1] West A G, Patrickson S J, Ehleringer J R. Water extraction times for plant and soil materials used in stable isotope analysis. *Rapid Commun Mass Sp* 2006; 20(8):1317-1321.
- [2] Peters L I, Yakir D. A direct and rapid leaf water extraction method for isotopic analysis. *Rapid Commun Mass Sp* 2008; 22(18):2929-2936.

- [3] Orlowski N, Pratt D L, McDonnell J J. Intercomparison of soil pore water extraction methods for stable isotope analysis. *Hydrol Process* 2016; 30(19):3434-3449.
- [4] Sprenger M, Herbstritt B, Weiler M. Established methods and new opportunities for pore water stable isotope analysis. *Hydrol Process* 2015; 29(25):5174-5192.
- [5] Song X, Barbour M M. Leaf water oxygen isotope measurement by direct equilibration. *New Phytol* 2016; 211(3):1120-1128.
- [6] Yang Q, Xiao H, Zhao L, Zhou M, Li C, Cao S. Stable isotope techniques in plant water sources a review. *Sciences in Cold and Arid Regions* 2010; 2(2):0112-0122.
- [7] McDonnell J J. The two water worlds hypothesis ecohydrological separation of water between trees and streams. *WIREs: Water* 2014; 1(4):323-329.
- [8] Orlowski N, Breuer L, McDonnell J J. Critical issues with cryogenic extraction of soil water for stable isotope analysis. *Ecohydrology* 2016; 9(1):1-5.
- [9] Hendry M J, Schmeling E, Wassenaar L I, Barbour S L, Pratt D. Determining the stable isotope composition of pore water from saturated and unsaturated zone core: Improvements to the direct vapour equilibration laser spectrometry method. *Hydrol Earth Syst Sc* 2015; 19(11):4427-4440.
- [10] Wassenaar L I, Hendry M J, Chostner V L, G.P. L. High resolution pore water  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  measurements by  $\text{H}_2\text{O}(\text{liquid})\text{-H}_2\text{O}(\text{vapor})$  equilibration laser spectroscopy. *Envir Sci Tech* 2008; 42(24):9262-9267.
- [11] Bottcher G, Brumsack H J, Heinrichs H, Pohlmann M. A new high pressure squeezing technique for pore fluid extraction from terrestrial soils. *Water Air Soil Poll* 1997; 94(3):289-296.
- [12] Orlowski N, Frede H G, Brüggemann N, Breuer L. Validation and application of a cryogenic vacuum extraction system for soil and plant water extraction for isotope analysis. *J Sensors* 2013; 2(2):179-193.
- [13] Koeniger P, Marshall J D, Link T, Mulch A. An inexpensive, fast, and reliable method for vacuum extraction of soil and plant water for stable isotope analyses by mass spectrometry. *Rapid Commun Mass Sp* 2011; 25(20):3041-3048.
- [14] Munksgaard N C, Cheesman A W, Wurster C M, Cernusak L A, Bird M I. Microwave extraction-isotope ratio infrared spectroscopy (me-iris): A novel technique for rapid extraction and in-line analysis of  $\delta^{18}\text{O}$  and  $\delta^2\text{H}$  values of water in plants, soils and insects. *Rapid Commun Mass Sp* 2014; 28(20):2151-2161.
- [15] Schmidt M, Maseyk K, Lett C, Biron P, Richard P, Bariac T, Seibt U. Concentration effects on laser-based  $\delta^{18}\text{O}$  and  $\delta^2\text{H}$  measurements and implications for the calibration of vapour measurements with liquid standards. *Rapid Commun Mass Sp* 2010; 24(24):3553-3561.
- [16] Leen J B, Berman E S F, Liebson L, Gupta M. Spectral contaminant identifier for off-axis integrated cavity output spectroscopy measurements of liquid water isotopes. *Rev Sci Instrum* 2012; 83(4)
- [17] West A G, Goldsmith G R, Brooks P D, Dawson T E. Discrepancies between isotope ratio infrared spectroscopy and isotope ratio mass spectrometry for the stable isotope analysis of plant and soil waters. *Rapid Commun Mass Sp* 2010; 24(14):1948-1954.
- [18] Brand W A, Geilmann H, Crosson E R, Rella C W. Cavity ring-down spectroscopy versus high-temperature conversion isotope ratio mass spectrometry; a case study on

- delta(2)h and delta(18)o of pure water samples and alcohol/water mixtures. *Rapid Commun Mass Sp* 2009; 23(12):1879-1884.
- [19] Morrison J, Brockwell T, Merren T, Fourel F, Phillips A M. On-line high precision stable hydrogen isotopic analyses on nanoliter water samples. *Anal Chem* 2001; 73(15):3570–3575.
- [20] Craig H. Standard for reporting concentration of deuterium and oxygen-18 in natural waters. *Science* 1961; 133(3467):1833-1834.
- [21] Epstein S, Mayeda T. Variation of o18 content of waters from natural sources. *Geochim Cosmochim Ac* 1953; 4(5):213-224.
- [22] Coplen T B. Guidelines and recommended terms for expression of stable-isotope-ratio and gas-ratio measurement results. *Rapid Commun Mass Sp* 2011; 25(17):2538-2560.
- [23] Wassenaar L I, Ahmad M, Aggarwal P, van Duren M, Poltenstein L, Araguas L, Kurttas T. Worldwide proficiency test for routine analysis of delta2h and delta18o in water by isotope-ratio mass spectrometry and laser absorption spectroscopy. *Rapid Commun Mass Sp* 2012; 26(15):1641-1648.
- [24] CoreTeam R, 3.3.2 ed., R Foundation for Statistical Computing, Vienna, Austria., **2013**.
- [25] Shapiro S S, Wilk M B. An analysis of variance test for normality. *Biometrika* 1965; 52(3/4):591-611.
- [26] Ghasemi A, Zahediasl S. Normality tests for statistical analysis: A guide for non-statisticians. *Int J Endocrinol Metab* 2012; 10(2):486-489.
- [27] Kruskal W H, Wallis W A. Use of ranks in one-criterion variance analysis. *J Am Stat Assoc* 1952; 47(260):583-621.
- [28] McKight P E, Najab J, in *The corsini encyclopedia of psychology*, John Wiley & Sons, Inc., **2010**.
- [29] Dunn O J. Multiple comparisons using rank sums. *Technometrics* 1964; 6(3):241-252.
- [30] Benjamini Y, Hochberg Y. Controlling the false discovery rate a practical and powerful approach to multiple testing. *J Roy Stat Soc B Met* 1995; 57(1):289-300.
- [31] Mann H B, Whitney D R. On a test of whether one of two random variables is stochastically larger than the other. *Ann Math Stat* 1947; 18(1):50-60.
- [32] Fay M P, Proschan M A. Wilcoxon-mann-whitney or t-test? On assumptions for hypothesis tests and multiple interpretations of decision rules. *Stat Surv* 2010; 4(1-39).
- [33] Fogel M L, Cifuentes L A, in *Organic geochemistry. Topics in geobiology, Vol. 11* (Eds.: M. H. Engel, S. A. Macko), Springer, Boston, MA., **1993**.
- [34] Martin-Gomez P, Barbeta A, Voltas J, Penuelas J, Dennis K, Palacio S, Dawson T E, Ferrio J P. Isotope-ratio infrared spectroscopy: A reliable tool for the investigation of plant-water sources? *New Phytol* 2015; 207(3):914-927.
- [35] Hendry M J, Richman B, Wassenaar L I. Correcting for methane interferences on delta2h and delta18o measurements in pore water using h2oliquid-h2ovapor equilibration laser spectroscopy. *Anal Chem* 2011; 83(14):5789-5796.
- [36] Scrimgeour C M. Measurement of plant and soil water isotope composition by direct equilibration methods. *J Hydrology* 1995; 172(1-4):261-274.
- [37] Vendramini P F, Sternberg L d S L. A faster plant stem-water extraction method. *Rapid Commun Mass Sp* 2008; 21(2):164-168.

- [38] Volkmann T H M, Kühnhammer K, Herbstritt B, Gessler A, Weile M. A method for in situ monitoring of the isotope composition of tree xylem water using laser spectroscopy. *Plant Cell Environ* 2016; 39(9):2055-2063.
- [39] Figueroa-Johnson M A, Tindall J A, Friedel M. A comparison of  $\delta^{18}\text{O}$  composition of water extracted from suction lysimeters, centrifugation, and azeotropic distillation. *Water Air Soil Poll* 2007; 184(1-4):63-75.

## 2.11 Tables and Figures

**Table 2.1.** Means and standard deviations (sd) of extracted  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  ratios from spring wheat samples for four different plant portions: head, leaf, stem, and root crown, measured via OA-ICOS and IRMS. (n.v.) indicates no data for this sample.

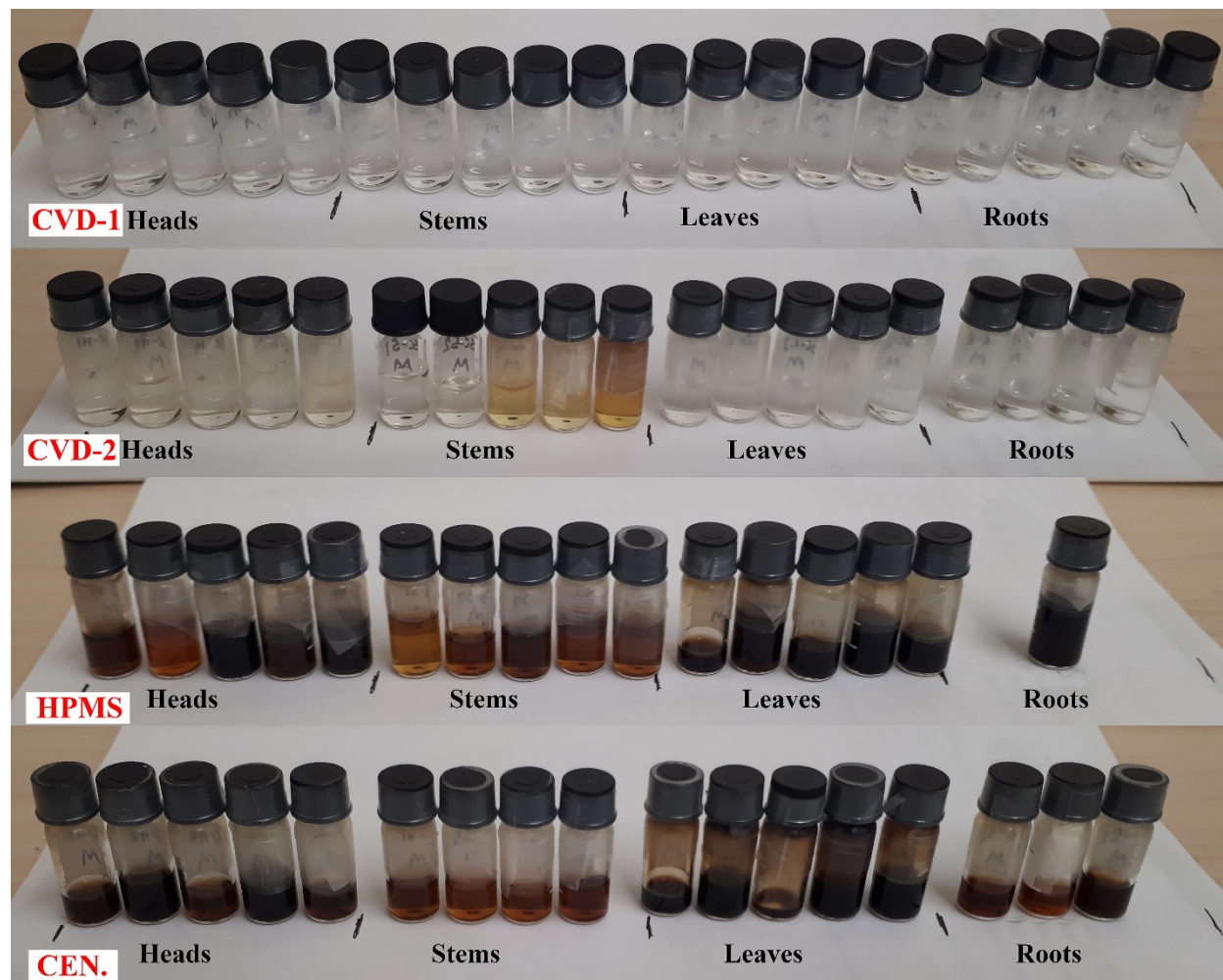
	OA-ICOS															
	Head				Leaf				Stem				Root Crown			
	$\delta^2\text{H}$ [‰]		$\delta^{18}\text{O}$ [‰]		$\delta^2\text{H}$ [‰]		$\delta^{18}\text{O}$ [‰]		$\delta^2\text{H}$ [‰]		$\delta^{18}\text{O}$ [‰]		$\delta^2\text{H}$ [‰]		$\delta^{18}\text{O}$ [‰]	
	Mean	sd	Mean	sd	Mean	sd	Mean	sd	Mean	sd	Mean	sd	Mean	sd	Mean	sd
<b>CVD-1</b>	-83.12	2.19	-0.09	0.31	-80.11	2.81	-1.12	1.02	-125.60	1.01	-12.59	0.34	-115.40	0.93	-11.95	0.34
<b>CVD-2</b>	-74.90	2.83	0.02	0.82	-74.41	1.50	1.36	1.75	-112.99	15.70	-5.15	8.53	-107.73	2.61	-9.45	1.25
<b>DVE</b>	-64.19	6.92	3.83	3.41	-64.96	1.48	0.98	0.65	-113.82	4.33	-11.32	0.81	-99.39	3.72	-9.20	0.64
<b>ME-OA-ICOS</b>	-37.32	5.50	5.78	1.27	-33.86	13.70	1.99	2.91	-95.93	6.72	-11.68	1.70	-91.14	5.92	-6.97	1.74
	IRMS															
	Head				Leaf				Stem				Root Crown			
	$\delta^2\text{H}$ [‰]		$\delta^{18}\text{O}$ [‰]		$\delta^2\text{H}$ [‰]		$\delta^{18}\text{O}$ [‰]		$\delta^2\text{H}$ [‰]		$\delta^{18}\text{O}$ [‰]		$\delta^2\text{H}$ [‰]		$\delta^{18}\text{O}$ [‰]	
	Mean	sd	Mean	sd	Mean	sd	Mean	sd	Mean	sd	Mean	sd	Mean	sd	Mean	sd
<b>CVD-1</b>	-81.79	1.28	-1.10	0.41	-82.08	2.87	-1.88	1.05	-127.28	2.35	-13.03	0.34	-115.58	0.86	-12.26	0.35
<b>CVD-2</b>	-77.20	3.51	3.59	4.39	-80.85	2.22	-1.14	1.44	-120.95	3.24	-9.72	3.04	-107.57	1.56	-5.16	4.36
<b>HPMS</b>	-67.40	4.24	-1.34	0.80	-76.36	6.92	-3.91	0.58	-107.69	4.23	-14.95	0.95	-101.32	n.v	-12.69	n.v
<b>Centrifugation</b>	-79.12	0.88	-2.38	0.53	-66.31	5.89	-0.07	0.30	-99.89	3.15	-11.53	0.51	-93.17	6.78	-8.99	1.45

**Table 2.2.** Results of methanol and ethanol content analysis by GC-FID of liquid analyte from all extraction systems that produced liquid analyte. A result of < 5 signifies that methanol or ethanol content was below detection level. N.v signifies no sample was supplied for analysis.

<b>Extraction Method and Plant Portion</b>	<b>Ethanol (ug/mL)</b>	<b>Methanol (ug/mL)</b>
CVD-1 – Head	4700	56
CVD-2 – Head	22000	690
HPMS – Head	22000	1900
Centrifuge – Head	30000	1100
CVD-1 – Leaf	150	44
CVD-2 – Leaf	7400	530
HPMS – Leaf	8700	870
Centrifuge – Leaf	1700	690
CVD-1 – Stem	1700	30
CVD-2 – Stem	8200	1100
HPMS – Stem	16000	1290
Centrifuge – Stem	15000	1400
CVD-1 – Root	<5	<5
CVD-2 – Root	670	190
HPMS – Root	2800	120
Centrifuge – Root	n.v.	n.v.

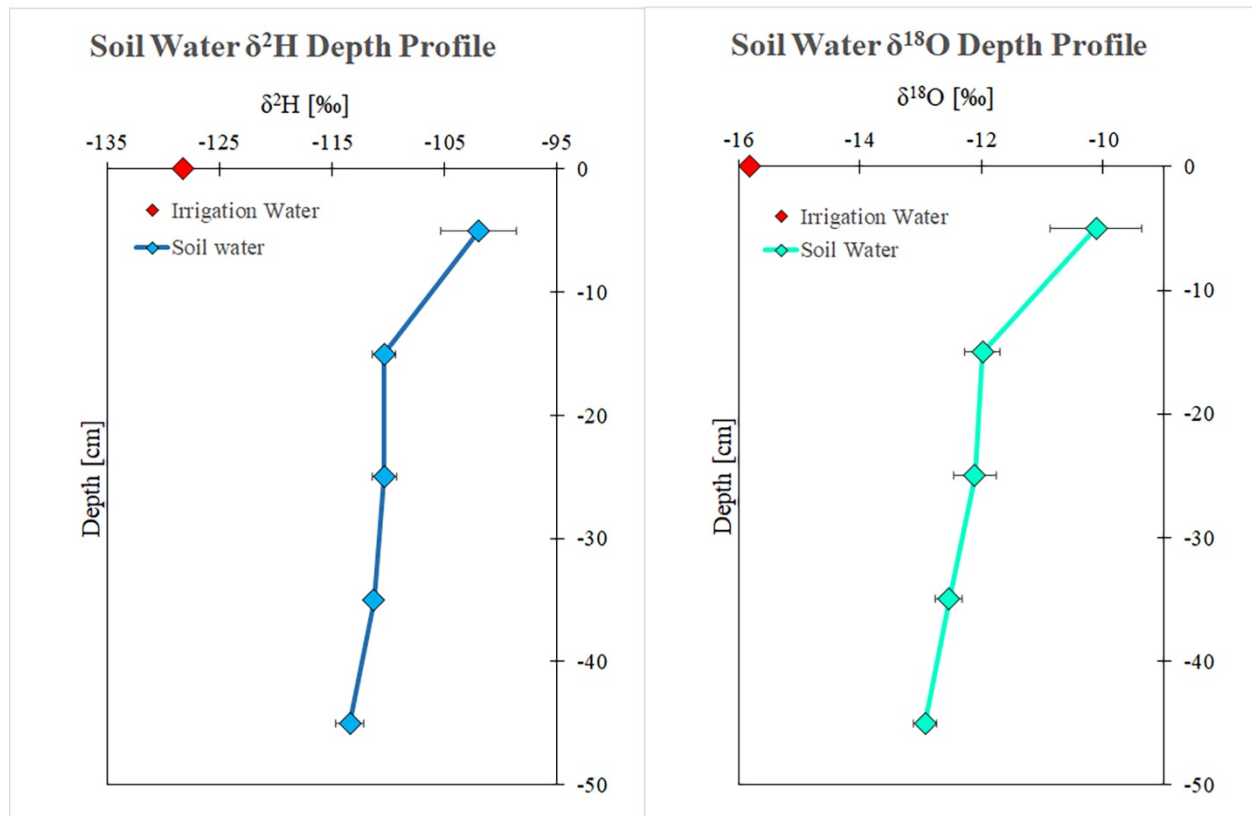


**Figure 2.1.** Photographic compilation of extracted analyte from cryogenic vacuum distillation: CVD-1 and CVD-2, high pressure mechanical squeezing (HPMS), and centrifugation (CEN), showing colour variations linked to co-extracted organics. Note: photos were taken after subsampling for analysis occurred, so volumes are not representative of total collected water.

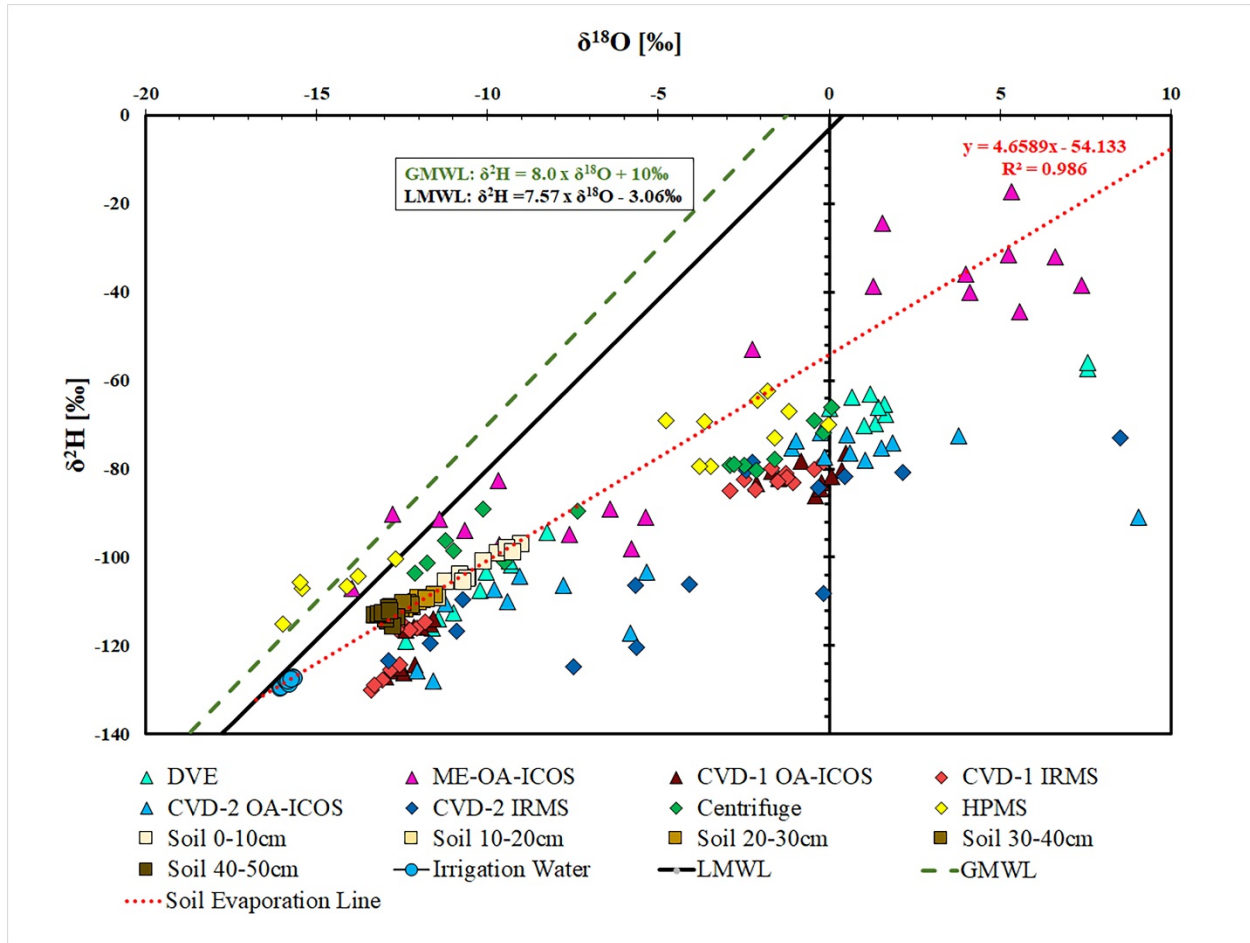


**Figure 2.2.** Stable isotope means and standard deviations (sd) of extracted soil pore water (n=10/depth), grouped by significantly different layers ( $p \leq 0.05$ ), with associated soil water stable isotope depth profiles. Water extracted via HPMS and analyzed via OA-ICOS.

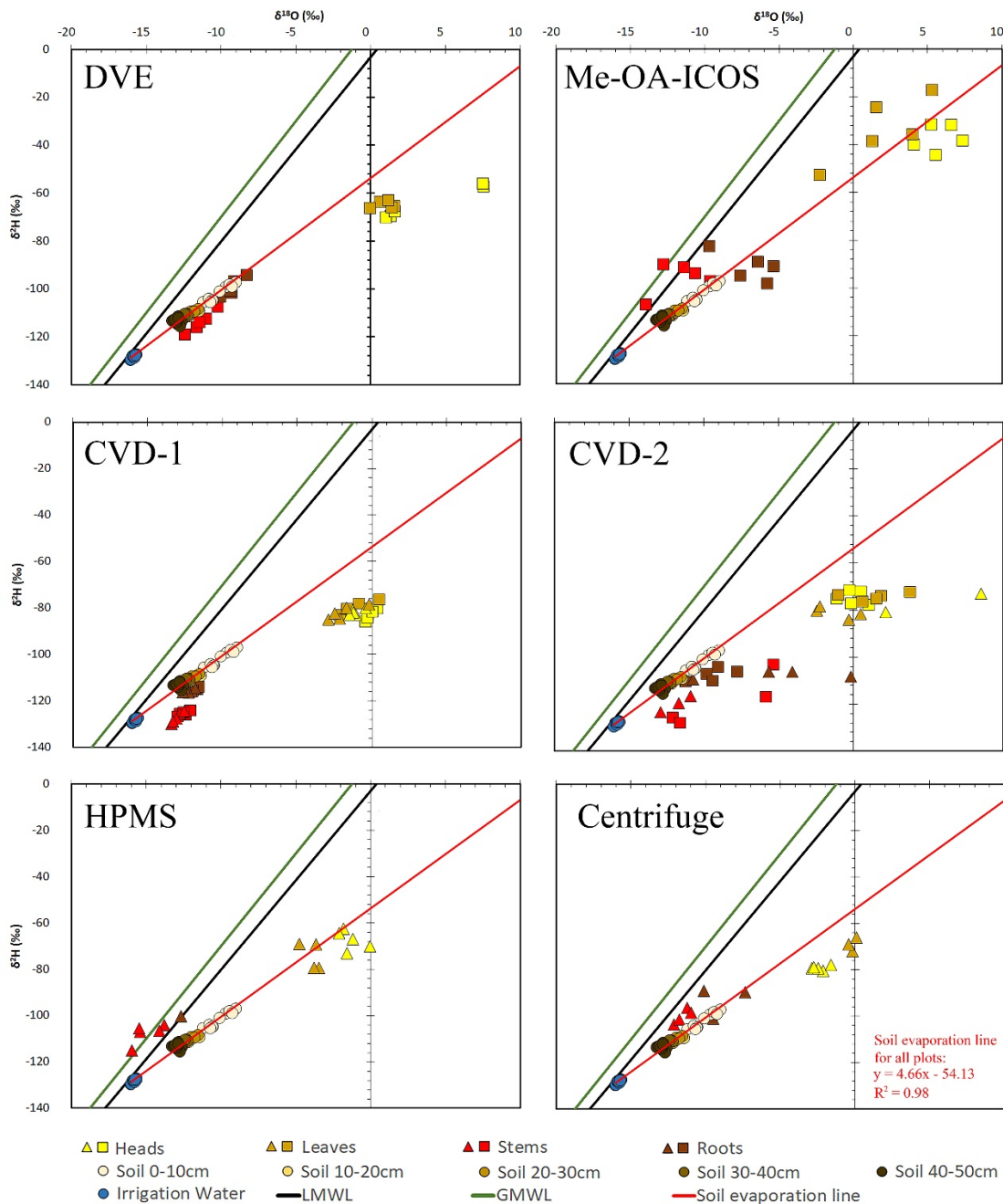
Depth (cm)	Soil Water Stable Isotope Values			
	$\delta^2\text{H}$ [‰]		$\delta^{18}\text{O}$ [‰]	
	Mean	sd	Mean	sd
0 -10	-101.97	3.37	-10.12	0.75
10-20	-110.38	1.02	-11.99	0.30
20-30	-110.37	1.10	-12.11	0.35
30-40	-111.27	0.61	-12.53	0.22
40-50	-113.43	1.21	-12.92	0.19



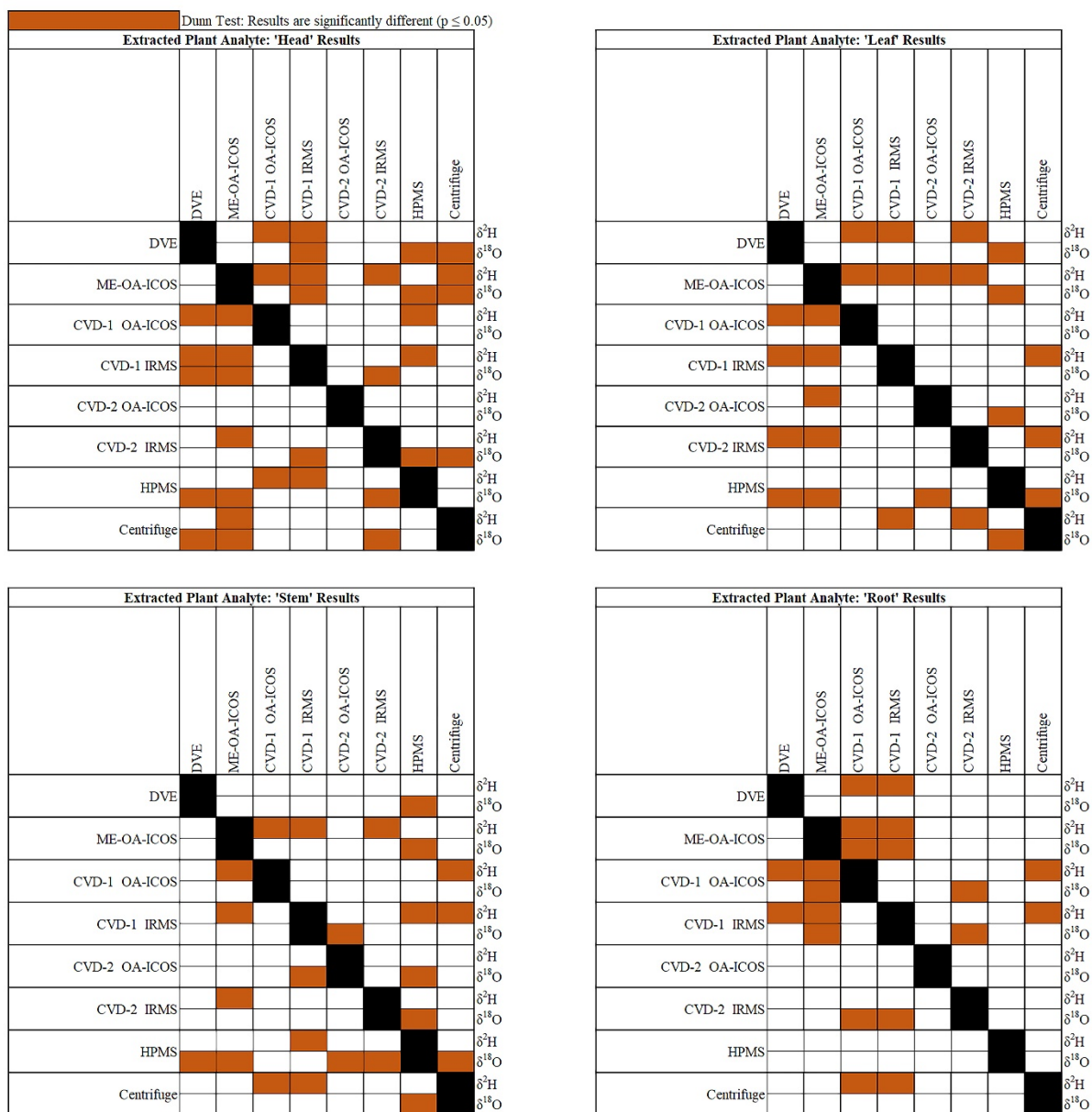
**Figure 2.3.** Dual isotope plot of all results: irrigation water, extracted soil water (via HPMS), and liquid analyte extracted from plant samples for all methods of extraction-analysis. Cryogenic vacuum distillation system results (CVD-1 and CVD-2) are grouped by analysis system used (OA-ICOS and IRMS).



**Figure 2.4.** Dual isotope plots of extracted plant analyte for all methods of extraction. DVE, ME-OA-ICOS, CVD-1, and CDV-2 analytes were analyzed via OA-ICOS (square markers). HPMS, centrifuge, CVD-1, and CVD-2 analytes were analyzed via IRMS (triangle markers). Extreme outliers were removed from the centrifugation results (n=1, leaf), the CVD-2-IRMS results (n=2, 1, 2: head, leaf, stem respectively) and the CVD-2 OA-ICOS results (n=1, stem).



**Figure 2.5.** Collection of statistical results detailing significant differences ( $p \leq 0.05$ ) between extraction methods per plant portion extracted for  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$ , produced by the post-hoc Dunn test utilizing the Benjamini-Hochberg adjustment to prevent false discovery rates.



**Table 2.S1.** Compilation of soil characteristics. Particle size analysis and clay mineralogy analyzed by X-ray powder diffraction. Clay chemical composition by X-ray fluorescence.

Particle Size Analysis		
Classification	Grain Size (mm)	Soil Fraction (%)
Gravel	> 4.75	0.73
Coarse Sand	2 - 4.75	6.70
Medium Sand	0.425 - 2.00	30.09
Fine Sand	0.075 - 0.425	52.82
Silt	0.005 - 0.075	6.66
Clay	< 0.005	2.99
	Total:	100.00

Clay Fraction Characteristics			
ID and Abundance		Chemical Composition	
Mineral	(wt%)	Component	(wt%)
Quartz [SiO <sub>2</sub> ]	53.10	Na <sub>2</sub> O	0.71
Dolomite [CaMgC <sub>2</sub> O <sub>6</sub> ]	1.40	MgO	1.1
Calcite [CaCO <sub>3</sub> ]	1.80	Al <sub>2</sub> O <sub>3</sub>	9.66
Albite [(Na <sub>0.821</sub> Ca <sub>0.179</sub> ) (Al <sub>1.179</sub> Si <sub>2.821</sub> ) O <sub>8</sub> ]	22.60	SiO <sub>2</sub>	62.48
Microcline [K(Si <sub>3</sub> Al)O <sub>8</sub> ]	14.30	P <sub>2</sub> O <sub>5</sub>	0.24
Hematite [Fe <sub>2</sub> O <sub>3</sub> ]	4.00	K <sub>2</sub> O	2.02
Clinochlore [Mg <sub>5.1</sub> Al <sub>1.2</sub> Cr <sub>0.7</sub> Si <sub>3</sub> O <sub>18</sub> H <sub>8</sub> ]	2.70	CaO	3.11
		TiO <sub>2</sub>	0.63
		MnO	0.08
		Fe <sub>2</sub> O <sub>3</sub>	3.36
		S	0.06

**Table 2.S2.** Irrigation water characteristics. Major ions analyzed by inductively coupled plasma (ICP) atomic emission spectroscopy.

<b>Irrigation Water Characteristics</b>	
$\delta^2\text{H}$ (‰):	-128.25 (SD: 0.65, n=18)
$\delta^{18}\text{O}$ (‰):	-15.80 (SD: 0.11, n=18)

<b>Inorganics</b>		
<b>Analyte</b>	<b>Units</b>	<b>Measurement</b>
pH	-	8.35
Specific conductivity	uS/cm	529
Sum of ions	mg/L	402
Total alkalinity	mg/L	144
Total hardness	mg/L	196
Bicarbonate	mg/L	171.00
Carbonate	mg/L	2
Chloride	mg/L	14
Hydroxide	mg/L	<1
P. alkalinity	mg/L	2

<b>Analysis by ICP</b>		
Nitrate	mg/L	0.65
Fluoride	mg/L	0.64
Calcium	mg/L	44.00
Magnesium	mg/L	21.00
Potassium	mg/L	4.90
Sodium	mg/L	34.00
Sulfate	mg/L	110.00

## Chapter 3: Further Experiments Comparing Direct Vapour Equilibration and Cryogenic Vacuum Distillation for Plant Water Stable Isotope Analysis

Cody Millar <sup>1</sup>, Dyan Pratt <sup>1</sup>, David J. Schneider <sup>2</sup>, Geoff Koehler<sup>3</sup> and Jeffrey J. McDonnell <sup>1</sup>.

1. Global Institute for Water Security, School of Environment and Sustainability, University of Saskatchewan, 11 Innovation Boulevard, Saskatoon, SK, S7N 3H5, Canada
2. Global Institute for Food Security, University of Saskatchewan, 110 Gymnasium Place, Saskatoon, SK, S7N 4J8, Canada.
3. Environment and Climate Change Canada, National Hydrology Research Centre. 11 Innovation Boulevard, Saskatoon, SK, S7N 3H5, Canada

Status: Manuscript in preparation to be submitted as a Letter to the Editor: Rapid Communications in Mass Spectrometry

### 3.1 Letter to the Editor

Dear Editor,

Recent work by Millar *et al.* <sup>[1]</sup> showed significant differences in the isotopic composition of analyte extracted by six unique methods of plant water extraction-analysis. Here we report a short follow-up experiment to further explore the systematic differences between the cryogenic vacuum distillation method (based on Koeniger *et al.* <sup>[2]</sup>), hereafter called CVD-2 as in Millar *et al.* <sup>[1]</sup>, and the direct vapour equilibration method (based on Wassenaar *et al.* <sup>[3]</sup> and <sup>[4]</sup>), hereafter called DVE.

Millar *et al.* <sup>[1]</sup> found that DVE was the extraction method most likely to be accessing the transpiration stream within wheat samples utilizing stable isotope analysis. This was in part because DVE does not rely on induced pressure or temperature changes to extract a vapour-analyte for analysis, and instead utilizes equilibration of plant tissue constrained water with dry headspace air in the sample container. Thus, the bulk of the analyte isotopic signal in the headspace will be from locations in the plant where water potential is highest: the transpiration stream. Further, Millar *et al.* <sup>[1]</sup> posit that since the DVE analysis step occurs at room temperature, only small volumes of organic compounds would be present in the vapour-analyte



headspace. This would thus limit interference during spectrometric isotopic analysis via off-axis integrated cavity output spectroscopy (OA-ICOS). We use the term analyte over that of plant water to refer to the liquid or vapour produced by the tested extraction-analysis approaches as there is potential in all extraction approaches to co-extract various volumes of organic compounds, thus the analyte cannot be thought of as purely ‘plant water’.

Many ecohydrological studies are interested in the isotopic signature of water in the plant transpiration stream <sup>[5, 6]</sup>. Systems like CVD-2 that extract up to 99% of the plant’s internal liquid content are not only accessing the transpiration stream, but also; intra cellular water, organelle constrained water, and organic compound constrained <sup>1</sup>H, <sup>2</sup>H, <sup>16</sup>O, and <sup>18</sup>O <sup>[1]</sup>. The isotopic signals of these other plant water compartments may be dominated by soluble organic compound content unrelated to the transpiration stream. Unexpectedly, Millar *et al.* <sup>[1]</sup> found that the stable isotope ratios ( $\delta^2\text{H}$ :  $^2\text{H}/^1\text{H}$ ,  $\delta^{18}\text{O}$ :  $^{18}\text{O}/^{16}\text{O}$ ) of the analyte extracted by the CVD-2 system were not significantly different at a 95% confidence interval from the isotopic results of the DVE system, except for the leaf analyte  $\delta^2\text{H}$  results. This did not fit with the working explanation of Millar *et al.* <sup>[1]</sup> for why there were differences between the isotopic composition of analytes produced by an equilibration method (DVE) and a bulk extraction method (CVD-2). That is, that the co-extracted organic compounds present in the analyte produced by the CVD-2 system would have skewed its isotopic results, making these results not representative of the plants transpiration stream isotopic signal. In fact, the CVD-2 system co-extracted substantial amounts of methanol (MeOH) and ethanol (EtOH) in the Millar *et al.* <sup>[1]</sup> study, which are known to cause errors in isotope ratio infrared spectrometry (IRIS) based analysis systems like OA-ICOS and may even modify isotope ratio mass spectrometry (IRMS) results if in high enough concentrations. <sup>[7-10]</sup>.

In this letter to the editor, we ask the following questions: Was the similarity between DVE and CVD-2 results in Millar *et al.* <sup>[1]</sup> due small sample sizes and thus lack of statistical power? Thus, will an increased number of samples for comparison between DVE and CVD-2 improve our understanding of method differences? Did co-extracted organic contamination cause errors in the isotopic analysis of analyte produced by the CVD-2 system? That is, did the organic contamination shift the CVD-2 results to appear statistically similar to the DVE results? We

believe it is unlikely that co-extracted organic contamination alone was responsible for the lack of difference between CVD-2 and DVE found in Millar *et al.* <sup>[1]</sup> as the CVD-2 extracts were analyzed by both IRMS and OA-ICOS analysis systems. IRMS analysis is typically used for plant extracted analytes to limit problems related to co-extracted organic compounds. The CVD-2 isotopic results produced by IRMS analysis should therefore have little interference from the presence of organic contaminants. Indeed, Millar *et al.* <sup>[1]</sup> found substantial differences between the CVD-2 stable isotope results returned by OA-ICOS analysis and those returned by IRMS analysis, indicating the impacts of organic contamination on OA-ICOS analysis. The more likely explanation for why the CVD-2 and DVE results were not significantly different was due to the small sample sizes used in the Millar *et al.* <sup>[1]</sup> inter-method comparison.

We use the phrase extraction-analysis to encompass both the CVD-2 extraction system and the DVE analysis approach. The CVD-2 system first *extracts* a liquid analyte from the plant, which is then sent for analysis via appropriate isotopic method. The DVE system is a measurement of isotopic values through direct *analysis* of a vapour analyte filled headspace above the plant sample of interest. To improve statistical understanding of the differences between DVE and CVD-2 Millar *et al.* <sup>[1]</sup> recommended further study with a higher ‘n’ per method of extraction-analysis. We carry out this improvement here.

Figure 1 shows spring wheat (*Triticum aestivum* L.) grown under controlled conditions to be utilized as a plant analyte source in this study. In this study a larger number of samples were collected than in Millar *et al.* <sup>[1]</sup> to improve statistical resolution. We used the same soil, containers, planting, and controlled irrigation approach as in Millar *et al.* <sup>[1]</sup> and refer the reader there for methodological details. However, in this study two containers of spring wheat were grown and only the root crown and first 5 cm of the leaf growth and differentiation zone (LGDZ) were collected for extraction-analysis. This plant portion was selected for its low co-extractable organic compound content <sup>[1]</sup> and its utility in indicating plant water sources <sup>[11]</sup>. Wheat samples were collected on a weekly basis once the seedlings had become established. The first sampling occurred two weeks after planting (‘week-1’ in the sampling regimen). For the following 8 weeks, a set of wheat samples were collected for DVE and CVD-2 extraction-analysis. During

each weekly sampling period a set of 8 individual plants were collected for each extraction-analysis method per growth container (container 1 (C1) and container 2 (C2)). We collected 32 total plant samples per week, split by extraction method and container (ex: 8 for DVE from C1, 8 for DVE from C2, 8 for CVD-2 from C1, and 8 for CVD-2 from C2). Each sample's LGDZ had the outer leaf layer removed as this component may have undergone transpirative enrichment. After extraction by CVD-2, the liquid analyte had its isotopic composition measured by IRMS. DVE samples were analyzed inline on a Los Gatos Research IWA-45EP OA-ICOS analyzer (Los Gatos Research Inc., San Jose, CA, USA).

Statistical analysis of the isotopic data was carried out with the software R 3.3.2.<sup>[12]</sup> Plant water stable isotope data was tested for normality using histograms, skewness, kurtosis, quantile-quantile plots, and the Shapiro-Wilk normality test<sup>[13, 14]</sup>. The plant water data, grouped by container (C1 and C2 separated), week sampled (week 1-8 separated), and extraction method (DVE v. CVD-2 separated), was found to be from normal distributions in some cases and from non-normal distributions in others. To check for extraction-analysis method differences we wanted to compare only samples that would be reasonable to compare to one another. Thus, we compared DVE to CVD-2 results on a weekly and container sampled from basis (ex: DVE results from samples collected on week-1 from C1 were compared to CVD-2 results from samples taken on week-1 from C1, similarly for C2, and for all eight sampling dates). For variance analysis between DVE and CVD-2 data, where both sets of data were from normal distributions, Student's T-test was applied<sup>[15]</sup> at a 95% confidence interval ( $p \leq 0.05$ ). For occasions where both sets of data were from non-normal distributions or one set of data was non-normal and the other was normal, the non-parametric Mann-Whitney-Wilcoxon test<sup>[16, 17]</sup> was used to determine significant differences between extraction-analysis methods ( $p \leq 0.05$ ).

Table 1 shows the mean and standard deviation (sd) of  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  results from each method of extraction-analysis, for each sampling week split by container (C1 and C2). For plant water  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  values from both containers, the CVD-2 extraction method consistently produced more negative values than those produced by the DVE analysis method. For C1  $\delta^2\text{H}$  values, the maximum difference between methods occurred during week-1 sampling: CVD-2 produced

analyte that was 34‰ more negative than DVE. The average difference between CVD-2 and DVE  $\delta^2\text{H}$  values from C1 across all weeks of sampling was -19‰. For C1  $\delta^{18}\text{O}$  values, the maximum difference between methods again occurred during week-1 sampling: CVD-2 produced results that were 7.3‰ more negative than DVE. The average difference between CVD-2 and DVE  $\delta^{18}\text{O}$  values from C1 across all weeks of sampling was -3.9‰. For C2, the maximum difference between CVD-2 and DVE  $\delta^2\text{H}$  results occurred in the week-7 samples, where CVD-2 produced values that were 33‰ more negative than DVE. The average difference between CVD-2 and DVE  $\delta^2\text{H}$  results across all weeks was -21‰. Similarly, for  $\delta^{18}\text{O}$  results, the maximum difference occurred in week-7 samples, where CVD-2 results were 7.2‰ more negative than DVE results. The average difference between CVD-2 and DVE results for C2 for all weeks was -4.2‰. The week-2  $\delta^{18}\text{O}$  results were the only time when the DVE method's weekly averaged results (-11.6‰, standard deviation (sd) = 4.0‰, n=8) were more negative than the CVD-2 results (-11.2‰, SD= 0.7, n=8). In most cases the sd of the eight extracted plant water results for each method of extraction tend to be relatively similar. However, in the week-2  $\delta^{18}\text{O}$  results, the DVE sd is substantially larger than the CVD-2 sd.

Figure 2 further highlights the relationship between the two method's results, wherein the CVD-2 system produced analytes whose stable isotope composition was consistently more negative than results produced by the DVE system for both  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  ratios. Figure 3 shows the results of variance testing by Student's t-test<sup>[15]</sup> and the Mann-Whitney-Wilcoxon rank sum test<sup>[16, 17]</sup>. Significant differences ( $p \leq 0.05$ ) were found between the stable isotope results produced by the DVE and CVD-2 methods for all weeks of sampling, except for C2 week-2  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  results. During that sampling period  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  results from both methods of extraction-analysis were not significantly different from one another.

The finding of significant differences between the isotopic composition of analyte returned from CVD-2 and DVE systems for nearly all weeks sampled and for both isotopic ratios of interest ( $\delta^2\text{H}$  and  $\delta^{18}\text{O}$ ) is contrary to the findings of Millar *et al.*<sup>[1]</sup>. Why are these two findings different? Co-extracted organic content in the samples could be a likely reason for these differences. Wu *et al.*<sup>[18]</sup> used advanced visualization techniques and reconstructive modelling to

show the water transport structures in the roots of wheat. They showed that the total area, and therefore water holding volume of the transport structures are relatively small compared to the area of all the other cellular structures. The cellular structures around the transport vessels will also be full of liquid, composed of water and other soluble organic compounds. As such, the transpiration stream isotopic signal only makes up a small portion of the total available liquid in any given plant portion. As noted in Millar *et al.* <sup>[1]</sup>, and strengthened by the findings of Wu *et al.* <sup>[18]</sup>, any extraction method that has a high extraction efficiency (e.g. CVD-2) will produce an analyte that will be strongly influenced by the soluble content of the non-transport related cells. Any compound with water solubility and vapourization temperatures above room temperature and below 200 °C (the extraction temperatures used in the CVD-2 approach) such as MeOH and EtOH will more readily become apparent in samples extracted via CVD-2 as compared to DVE, as compounds normally associated with plant cellular structures are not easily volatilized at room temperature. For this study, we expect MeOH and EtOH concentration of the analyte extracted from the plant portion (made up of the root crown and LGDZ) to be similar to what was seen in Millar *et al.* (2018) for those plant portions. We are predicting that the concentrations of these organic compounds would vary throughout the growing stages of the plant, increasing as plant energetic organic compounds (carbohydrates etc.) are created during photosynthesis and stored in the LGDZ for later mobilization during anthesis. Fogel and Cifuentes <sup>[19]</sup> showed that plants may be preferentially choosing <sup>1</sup>H over <sup>2</sup>H in the production of organic compounds. Were this the case in our wheat samples, then analyte produced by the CVD-2 approach, containing higher volumes of soluble organic compound content composed preferentially of <sup>1</sup>H dominated organic compounds could serve to drive isotopic results in a more negative direction for  $\delta^2\text{H}$  ratios (depleted in <sup>2</sup>H and enriched in <sup>1</sup>H). As we have not quantified MeOH and EtOH content for each sampling point we won't carry this assumption too far, however, rising concentrations of these compounds in the analyte produced by CVD-2 extraction may explain differences in standard deviations seen over the course of the experiment between the two extraction methodologies.

Another important consideration for explaining the potential differences between DVE and CVD-2 isotopic results, is related to the way in which the IRMS and OA-ICOS systems produce

stable isotope results. Simply put, IRMS approaches physically separate and then count the number of  $^1\text{H}$ ,  $^2\text{H}$ ,  $^{16}\text{O}$  and  $^{18}\text{O}$  atoms presents in a given analyte sample. Thus, IRMS approaches limit issues related to organic contamination in water samples since the volume of contaminant is typically relatively low compared to the volume of water in a given plant extracted sample. If contaminants are in high enough concentrations to cause errors, the size of the contamination effect is directly related to the concentration of the contaminant in the sample and its isotopic ratio <sup>[7]</sup>. In such situations, correction of the resulting isotope value can be made via mass balance correction, which utilizes the isotopic ratios of the water and contaminant and their respective molar fractions <sup>[7]</sup>. This requires that contaminant concentrations in the analyte be well defined. IRIS approaches on the other hand utilize highly specific photo absorption characteristics, relatively unique to water isotopologues ( $^1\text{H}^1\text{H}^{16}\text{O}$ ,  $^1\text{H}^2\text{H}^{16}\text{O}$ , and  $^1\text{H}^1\text{H}^{18}\text{O}$ ), to determine the isotopic ratios ( $\delta^2\text{H}$  ( $^2\text{H}/^1\text{H}$ ) and  $\delta^{18}\text{O}$  ( $^{18}\text{O}/^{16}\text{O}$ )) of the analyte (water and co-extracted organics). The presence of any organic contaminants in plant extracted analytes that contain OH bonds similar to those of water will thus share similar spectral features with water. This results in the well-studied error issues related to organic contamination and IRIS analysis approaches <sup>[7-10, 20]</sup>. Further, the specific range of the water absorption band of the electromagnetic (EM) spectrum being utilized in IRIS analysis is likely unique to each IRIS manufacturer and may even have variations between individual devices of the same manufacturer<sup>[7, 10]</sup>. Additionally, the mathematical fitting procedures translating absorption into stable isotope values are unique to each manufacturer.

In this study, the above points are relevant since we are comparing results produced by IRMS to OA-ICOS. We can therefore assume that the CVD-2 isotopic results are accurate representations of the analyte extracted by this system due to their analysis by IRMS: that is the bulk water pool of the wheat. For DVE OA-ICOS, if there are co-extracted organic contaminants sharing spectral features with water present in the analysis headspace, it may be that these OA-ICOS isotopic results are prone to some level of error due to spectral contamination. This could be another explanation for why we see differences in the DVE and CVD-2 results. However, if these plant organic compounds are preferentially built using lighter isotopes ( $^1\text{H}$  and  $^{16}\text{O}$ )<sup>[19]</sup> we would expect their presence in the headspace of the DVE to drive the results in a more negative

direction for  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  values (depleted in heavy isotopes  $^2\text{H}$  and  $^{18}\text{O}$  and enriched in lighter isotopes  $^1\text{H}$  and  $^{16}\text{O}$ ). However, we do not see this in our results. In fact, our DVE results are more positive for  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  values than CVD-2 results for all weeks of sampling. We therefore believe that limited co-extracted organics are present in the headspace of DVE samples, and that our isotopic results are thus representative of the transpiration stream water isotopic signal.

With a higher number of samples available for comparison in the present study, contrary to the findings of Millar *et al.* [1], we determined that there are significant differences in the stable isotope composition of analytes extracted by the CVD-2 system and isotopic results produced by DVE analysis. While the DVE approach is accessing water from location in the plant where water potential is highest: the transpiration stream, we cannot entirely rule out potential interference effects related to organic compound content in the analysis headspace of DVE samples, though we believe this problem to be limited with spring wheat samples. As organic compound content is well known to cause interference and errors in OA-ICOS analysis, further research is required to investigate organic compound detection tools during vapour analysis on OA-ICOS systems as well as identifying what compounds are present and quantifying their effects on isotopic results. Collaboration between isotope ratio infrared spectroscopy system manufacturers and research scientists may be necessary as components of this technology related to the return of isotopic results contains trade secrets and proprietary information. Our findings further indicate the importance of choosing the extraction-analysis approach best suited to accessing the water pools of interest for specific research campaigns. For studies interested in the transpiration stream, DVE could be a powerful tool for investigating plant water sourcing, as long as issues related to organic compound interference can be addressed. A potential future option would be to run DVE samples on IRMS, thereby limiting contamination issues.

### 3.2 Acknowledgements

Thanks are due to Kim Janzen and the McDonnell Watershed Hydrology Lab group. The collaboration of the Environment Canada Stable Isotope Laboratory in Saskatoon was

invaluable. This research was supported by an NSERC Discovery grant to JJM, and by the NSERC CREATE program.

### 3.3 Transition Statement

Chapter 3 has further improved our understanding of potential differences in stable isotopes results produced by the DVE and CVD-2 extraction-analysis approaches. This chapter highlights the need for further research of the effects that co-extracted organic compounds like methanol and ethanol could have on isotopic results returned by OA-ICOS analysis approaches. It also highlights the need for development of detection tools to indicate the presence of organic contaminants in the headspace of DVE samples, during vapour-mode analysis. This leads us to Chapter 4 wherein we investigate the effects of methanol and ethanol contamination on OA-ICOS and IRMS isotopic results.

### 3.4 Author Contributions

C. Millar designed the study; planted, maintained, and collected the wheat samples; carried out extraction-analysis of plant analyte samples; conducted all data analysis; wrote the manuscript and created the figures. D. Pratt provided consultation on experiment design, provided consultation on analyte extraction/analysis, and edited the manuscript. D. Schneider, G. Koehler and J.J. McDonnell edited the manuscript and provided critical feedback and insights into understanding results, as well assisting in development of the discussion section of the manuscript.

### 3.5 References

- [1] Millar C, Pratt D, Schneider D J, McDonnell J J. A comparison of extraction systems for plant water stable isotope analysis. *Rapid Commun Mass Sp* 2018; 32(13):1031-1044.
- [2] Koeniger P, Marshall J D, Link T, Mulch A. An inexpensive, fast, and reliable method for vacuum extraction of soil and plant water for stable isotope analyses by mass spectrometry. *Rapid Commun Mass Sp* 2011; 25(20):3041-3048.
- [3] Wassenaar L I, Hendry M J, Chostner V L, G.P. L. High resolution pore water  $\delta^{2}\text{H}$  and  $\delta^{18}\text{O}$  measurements by  $\text{H}_2\text{O}(\text{liquid})\text{-H}_2\text{O}(\text{vapor})$  equilibration laser spectroscopy. *Envir Sci Tech* 2008; 42(24):9262-9267.
- [4] Hendry M J, Schmeling E, Wassenaar L I, Barbour S L, Pratt D. Determining the stable isotope composition of pore water from saturated and unsaturated zone core:



- Improvements to the direct vapour equilibration laser spectrometry method. *Hydrol Earth Syst Sc* 2015; 19(11):4427-4440.
- [5] Brooks J R, Barnard H R, Coulombe R, McDonnell J J. Ecohydrologic separation of water between trees and streams in a mediterranean climate. *Nature Geoscience* 2010; 3(2):100-104.
  - [6] Evaristo J, McDonnell J J, Clemens J. Plant source water apportionment using stable isotopes: A comparison of simple linear, two-compartment mixing model approaches. *Hydrological Processes* 2017; 31(21):3750-3758.
  - [7] Brand W A, Geilmann H, Crosson E R, Rella C W. Cavity ring-down spectroscopy versus high-temperature conversion isotope ratio mass spectrometry; a case study on  $\delta(2)H$  and  $\delta(18)O$  of pure water samples and alcohol/water mixtures. *Rapid Commun Mass Sp* 2009; 23(12):1879-1884.
  - [8] West A G, Goldsmith G R, Brooks P D, Dawson T E. Discrepancies between isotope ratio infrared spectroscopy and isotope ratio mass spectrometry for the stable isotope analysis of plant and soil waters. *Rapid Commun Mass Sp* 2010; 24(14):1948-1954.
  - [9] Martin-Gomez P, Barbeta A, Voltas J, Penuelas J, Dennis K, Palacio S, Dawson T E, Ferrio J P. Isotope-ratio infrared spectroscopy: A reliable tool for the investigation of plant-water sources? *New Phytol* 2015; 207(3):914-927.
  - [10] Leen J B, Berman E S F, Liebson L, Gupta M. Spectral contaminant identifier for off-axis integrated cavity output spectroscopy measurements of liquid water isotopes. *Rev Sci Instrum* 2012; 83(4)
  - [11] Barnard R L, de Bello F, Gilgen A K, Buchmann N. The  $\delta 18O$  of root crown water best reflects source water  $\delta 18O$  in different types of herbaceous species. *Rapid Commun Mass Spectrom* 2006; 20(24):3799-3802.
  - [12] CoreTeam R, 3.3.2 ed., R Foundation for Statistical Computing, Vienna, Austria., **2013**.
  - [13] Shapiro S S, Wilk M B. An analysis of variance test for normality. *Biometrika* 1965; 52(3/4):591-611.
  - [14] Ghasemi A, Zahediasl S. Normality tests for statistical analysis: A guide for non-statisticians. *Int J Endocrinol Metab* 2012; 10(2):486-489.
  - [15] Student. The probable error of a mean. *Biometrika* 1908; 6(1-25).
  - [16] Mann H B, Whitney D R. On a test of whether one of two random variables is stochastically larger than the other. *Ann Math Stat* 1947; 18(1):50-60.
  - [17] Fay M P, Proschan M A. Wilcoxon-mann-whitney or t-test? On assumptions for hypothesis tests and multiple interpretations of decision rules. *Stat Surv* 2010; 4(1-39).
  - [18] Wu H, Jaeger M, Wang M, Li B, Zhang B G. Three-dimensional distribution of vessels, passage cells and lateral roots along the root axis of winter wheat (*triticum aestivum*). *Ann Bot* 2011; 107(5):843-853.
  - [19] Fogel M L, Cifuentes L A, in *Organic geochemistry. Topics in geobiology, Vol. 11* (Eds.: M. H. Engel, S. A. Macko), Springer, Boston, MA., **1993**.
  - [20] Schultz N M, Griffis T J, Lee X, Baker J M. Identification and correction of spectral contamination in  $2H/1H$  and  $18O/16O$  measured in leaf, stem, and soil water. *Rapid Commun Mass Spectrom* 2011; 25(21):3360-3368.
  - [21] Pham S V, Leavitt P R, McGowan S, Wissel B, Wassenaar L I. Spatial and temporal variability of prairie lake hydrology as revealed using stable isotopes of hydrogen and oxygen. *Limnol Oceanogr* 2009; 54(1):101-118.

**Table 3.1.** Means and standard deviations (sd) of  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  ratios from spring wheat samples, extracted by DVE and CVD-2 for container-1 and container-2 from all 8 weeks of sampling. N=8 for each method of extraction per week of sampling. DVE samples analyzed via OA-ICOS and CVD-2 samples analyzed via IRMS.

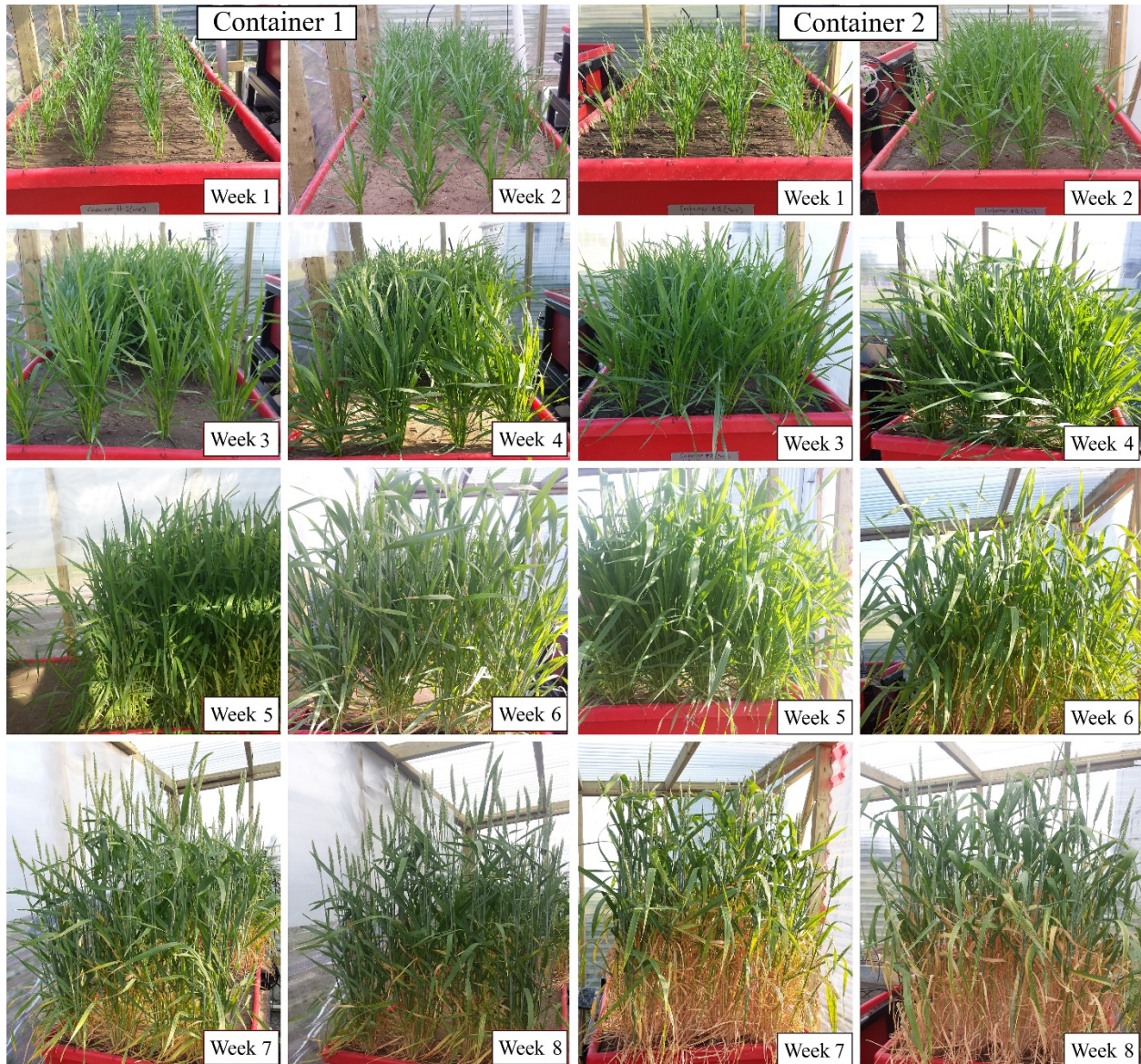
Container-1: $\delta^2\text{H}$ Results																
Extraction/Analysis Method	$\delta^2\text{H}$ value (‰)															
	Week 1		Week 2		Week 3		Week 4		Week 5		Week 6		Week 7		Week 8	
	Mean (‰)	sd (‰)	Mean (‰)	sd (‰)	Mean (‰)	sd (‰)	Mean (‰)	sd (‰)	Mean (‰)	sd (‰)	Mean (‰)	sd (‰)	Mean (‰)	sd (‰)	Mean (‰)	sd (‰)
DVE	-75.3	3.7	-89.5	6.4	-95.7	4.3	-96.9	7.6	-101.9	10.2	-102.5	8.6	-95.5	10.3	-110.1	7.0
CVD-2	-109.8	2.8	-104.6	1.9	-115.9	4.3	-116.5	3.2	-112.5	4.1	-118.3	2.2	-122.1	4.1	-121.0	3.5

Container-1: $\delta^{18}\text{O}$ Results																
Extraction/Analysis Method	$\delta^{18}\text{O}$ value (‰)															
	Week 1		Week 2		Week 3		Week 4		Week 5		Week 6		Week 7		Week 8	
	Mean (‰)	sd (‰)	Mean (‰)	sd (‰)	Mean (‰)	sd (‰)	Mean (‰)	sd (‰)	Mean (‰)	sd (‰)	Mean (‰)	sd (‰)	Mean (‰)	sd (‰)	Mean (‰)	sd (‰)
DVE	-4.4	1.3	-7.7	1.3	-8.9	0.5	-9.3	0.6	-9.8	1.1	-9.7	1.2	-8.6	1.7	-10.4	1.5
CVD-2	-11.6	0.6	-10.2	0.5	-12.9	0.7	-13.6	0.6	-12.5	1.2	-13.4	0.4	-13.3	1.0	-12.7	1.0

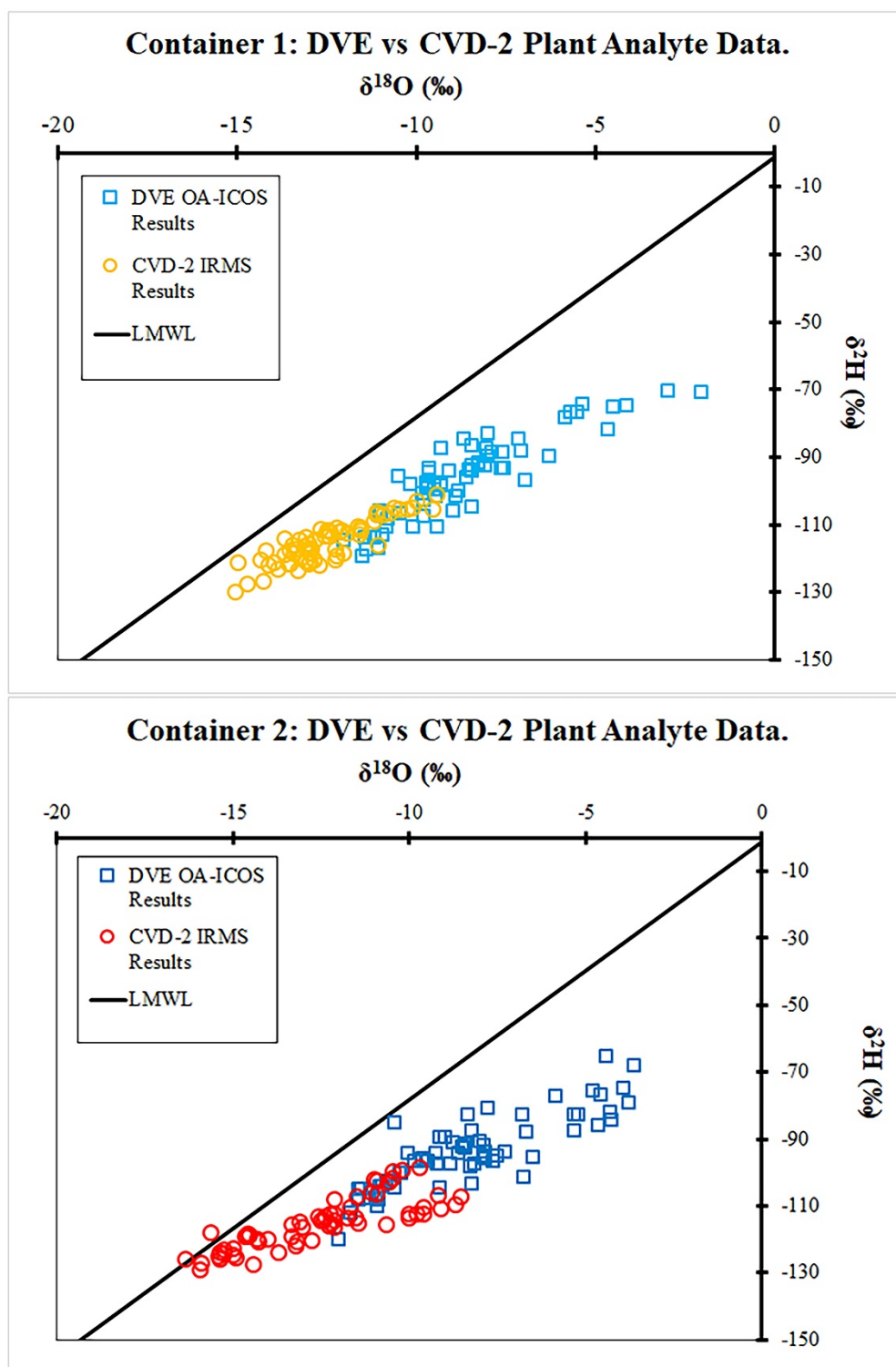
Container 2: $\delta^2\text{H}$ Results																
Extraction/Analysis Method	$\delta^2\text{H}$ value (‰)															
	Week 1		Week 2		Week 3		Week 4		Week 5		Week 6		Week 7		Week 8	
	Mean (‰)	sd (‰)	Mean (‰)	sd (‰)	Mean (‰)	sd (‰)	Mean (‰)	sd (‰)	Mean (‰)	sd (‰)	Mean (‰)	sd (‰)	Mean (‰)	sd (‰)	Mean (‰)	sd (‰)
DVE	-77.1	7.5	-102.5	7.9	-96.4	5.2	-108.0	5.5	-90.6	5.8	-95.1	5.8	-84.9	7.8	-96.3	4.0
CVD-2	-108.0	7.7	-104.9	4.4	-125.0	2.5	-120.4	3.0	-117.3	4.3	-115.5	2.8	-117.9	5.9	-110.9	3.0

Container 2: $\delta^{18}\text{O}$ Results																
Extraction/Analysis Method	$\delta^{18}\text{O}$ value (‰)															
	Week 1		Week 2		Week 3		Week 4		Week 5		Week 6		Week 7		Week 8	
	Mean (‰)	sd (‰)	Mean (‰)	sd (‰)	Mean (‰)	sd (‰)	Mean (‰)	sd (‰)	Mean (‰)	sd (‰)	Mean (‰)	sd (‰)	Mean (‰)	sd (‰)	Mean (‰)	sd (‰)
DVE	-5.1	1.2	-11.6	4.0	-9.4	0.9	-11.2	0.4	-9.0	0.9	-8.1	0.4	-4.9	1.0	-7.9	0.8
CVD-2	-11.4	1.2	-11.2	0.7	-15.5	0.6	-15.0	0.4	-13.7	1.0	-12.4	0.5	-12.1	1.7	-9.5	0.7

**Figure 3.1.** Photographic compilation of container 1 and container 2 spring wheat growth stages during each week of sampling. Note that water of the same isotopic composition was used during irrigation of both containers, but C2 received half the water of C1 during the last 4 weeks of the trial as part of an experiment being run in parallel to this one.

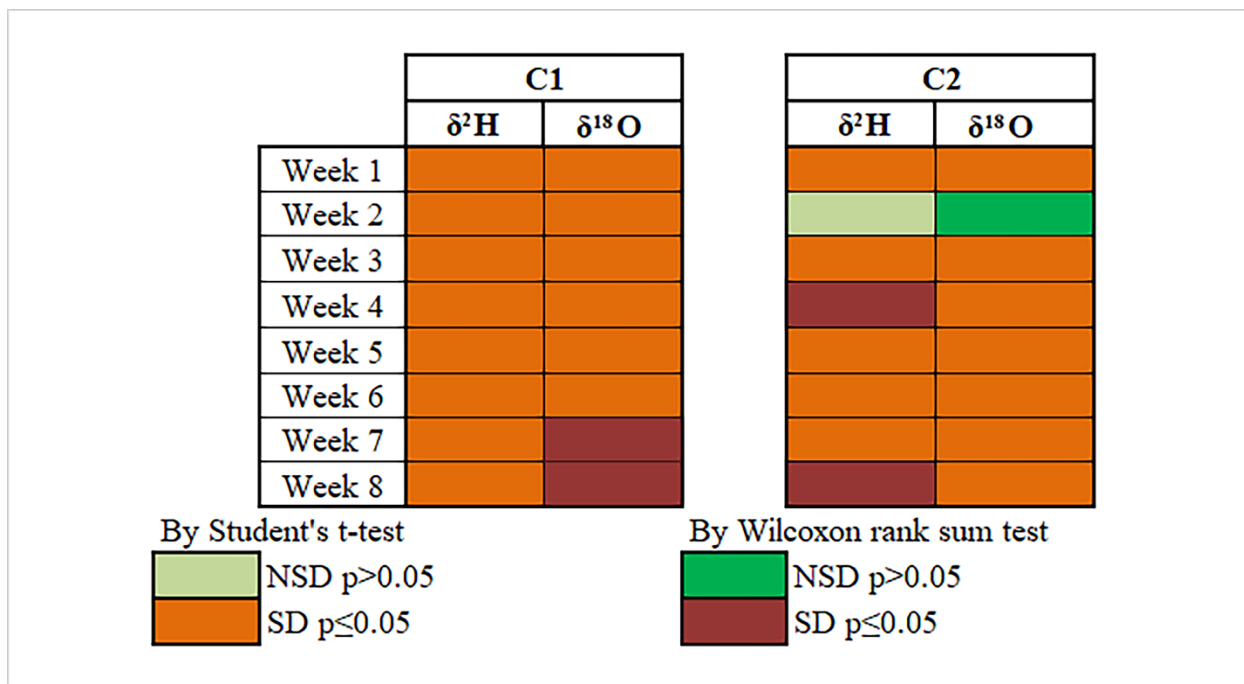


**Figure 3.2.** Dual isotope plots of extracted plant water  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  values from DVE and CVD-2 methods, for all weeks of sampling. Local meteoric water line (LMWL) equation:  $\delta^2\text{H} = 7.7 \times \delta^{18}\text{O} - 1.2\text{‰}$ <sup>[21]</sup>.





**Figure 3.3.** Results of variance testing between DVE and CVD-2 extraction-analysis system's returned  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  results for container-1 (C1) and container-2 (C2). When both sets of data were from normal distributions, data was compared using Student's t-test to find significant differences between extraction-analysis methods ( $p \leq 0.05$ ). For cases when one set of data was from a normal distribution and the other was from a non-normal distribution, or when both sets of data were from non-normal distributions the Wilcoxon rank sum test was used to determine significant differences between stable isotope results ( $p \leq 0.05$ ). Significantly different (SD). Not significantly different (NSD).



## **Chapter 4: Differences Between off-axis Integrated Cavity Output Spectroscopy and Isotope Ratio Mass Spectrometry for Organically Contaminated Samples in Liquid and Vapour Mode**

Cody Millar<sup>1</sup>, Kim Janzen<sup>1</sup>, David J Schneider<sup>2</sup>, Geoff Koehler<sup>3</sup>, Jeffrey J. McDonnell<sup>1</sup>.

1. Global Institute for Water Security, School of Environment and Sustainability, University of Saskatchewan, 11 Innovation Boulevard, Saskatoon, SK, S7N 3H5, Canada.
2. Global Institute for Food Security, University of Saskatchewan, 110 Gymnasium Place, Saskatoon, SK, S7N 4J8, Canada.
3. Environment and Climate Change Canada, National Hydrology Research Centre. 11 Innovation Boulevard, Saskatoon, SK, S7N 3H5, Canada

Status: Manuscript in preparation for publication submission to RCMS

### **4.1 Abstract**

**RATIONALE:** Co-extracted organic compounds such as methanol and ethanol are known to cause errors during stable isotope analysis of plant water via isotope ratio infrared spectrometry (IRIS). Additionally, research on two common IRIS methods has shown that the Los Gatos Research (LGR) off-axis isotope ratio spectrometry (OA-ICOS) method and Picarro's cavity ring down spectroscopy (CRDS) method produce significantly different results when exposed to the same organic compound contaminated plant water. Quantification of the effect of methanol and ethanol contamination on the stable isotope composition of water samples when analyzed via OA-ICOS is needed. Software for the detection of organic contamination during liquid analysis on OA-ICOS exists. However, tools for the detection of organic contamination during vapour-mode analysis via OA-ICOS are needed.

**METHODS:** We create a series of methanol-water and ethanol-water mixtures and analyze them for their stable isotope composition via liquid and vapour-mode on an LGR IWA-45EP OA-ICOS analyzer. We analyze the same mixtures via isotope ratio mass spectrometry, utilizing hydrogen reduction for  $\delta^2\text{H}$  ratio analysis and  $\text{CO}_2\text{-H}_2\text{O}$  equilibration for  $\delta^{18}\text{O}$  ratio analysis. We

test the Absorption plot and Spectra Fit Residuals plot, components of the OA-ICOS system, for their ability to detect organic contamination in water samples during vapour mode analysis.

**RESULTS:** For both  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  ratios, OA-ICOS results showed substantial deviations from IRMS and OA-ICOS reference water results for methanol-water mixtures at greater than 0.01% concentrations. Methanol-water mixtures showed deviations as large as 406‰ ( $\delta^2\text{H}$ ) and 261‰ ( $\delta^{18}\text{O}$ ) relative to the reference water isotopic composition when analysed in liquid-mode via OA-ICOS. Ethanol-water mixtures only showed noticeable deviations between OA-ICOS and reference water results at 5% and 10% concentrations. Deviations as large as -9.8‰ ( $\delta^2\text{H}$ ) and -4.1‰ ( $\delta^{18}\text{O}$ ) relative to the control water isotopic composition were measured during liquid analysis of ethanol-water mixtures on OA-ICOS. The Spectra Fit Residuals plot detected methanol contamination at concentrations  $\geq 0.01\%$ , and ethanol contamination at concentrations  $\geq 0.1\%$  in vapour-mode on OA-ICOS.

**CONCLUSION:** Our findings show that methanol causes far more error in isotopic results than ethanol contamination during spectrometric analysis. Unlike previous work that showed organic contamination caused CRDS results to skew negatively and OA-ICOS results to skew positively in the presence of organic contaminants, we show that methanol causes OA-ICOS results to skew positively, while ethanol causes OA-ICOS result to skew negatively. We show that the LGR Spectra Fit Residuals plot may be a useful tool for the detection of organic contaminated results during vapour-mode analysis.

## 4.2 Introduction

Analyte extracted from plants for stable isotope analysis are composed of plant water that commonly contains co-extracted soluble organic compounds. These organic compounds are known to cause substantial errors in returned isotopic results when that analyte is analyzed via isotope ratio infrared spectroscopy (IRIS) methods<sup>[1-4]</sup>. Therefore, plant analytes are typically analyzed by isotope ratio mass spectrometry (IRMS), wherein the size of the contamination effect is directly related to the concentration of the contaminant in the sample and its isotopic ratio<sup>[1]</sup>. In such situations, correction of the resulting isotope value can be made via mass

balance correction, which utilizes the isotopic ratios of the water and contaminant, and their respective molar fractions <sup>[1]</sup>. This requires that contaminant concentrations in the analyte be well defined.

IRIS methods include the Picarro (Picarro, Santa Clara, CA, USA) cavity ring down spectroscopy (CRDS) system and the Los Gatos Research (Los Gatos Research Inc., San Jose, CA, USA) off axis-integrated cavity output spectroscopy (OA-ICOS) system. IRIS analysis systems utilize highly specific photo absorption characteristics, relatively unique to water isotopologues ( $^1\text{H}^1\text{H}^{16}\text{O}$ ,  $^1\text{H}^2\text{H}^{16}\text{O}$ , and  $^1\text{H}^1\text{H}^{18}\text{O}$ ), to determine the isotopic ratios ( $\delta^2\text{H}$  ( $^2\text{H}/^1\text{H}$ ) and  $\delta^{18}\text{O}$  ( $^{18}\text{O}/^{16}\text{O}$ )) of the analyte (water and co-extracted organics)<sup>[1]</sup>. The two most commonly cited problematic organic contaminants present in extracted plant analytes are methanol [ $\text{CH}_3\text{OH}$ ] (MeOH) and ethanol [ $\text{C}_2\text{H}_6\text{O}$ ] (EtOH) <sup>[1, 2, 4]</sup>. The OH bonds of methanol and ethanol share similar spectral features with water, which is the suspected source of the interference during plant analyte stable isotope analysis via IRIS methods <sup>[1]</sup>.

Brand *et al.* <sup>[1]</sup> studied the effect of MeOH and EtOH contamination in lab prepared water-contaminant samples on a Picarro CRDS by comparing these results to those produced by thermal conversion/elemental analyzer IRMS (TC/EA-IRMS). They showed that MeOH contamination caused greater errors than EtOH contamination on CRDS isotopic results, but that both MeOH and EtOH contamination caused IRIS results to differ from IRMS results. West *et al.* <sup>[2]</sup> furthered this work by testing water extracted from 11 different plant species, and one organic soil on IRIS and IRMS methods. They found that for over half the samples, significant and substantial deviations occurred between IRIS and IRMS results, due to co-extracted organic contamination in the plant extracted analytes. Interestingly they showed that the CRDS and OA-ICOS systems produced significantly different results when exposed to the more strongly contaminated plant water samples.

While effects of organic contamination have been well studied on the CRDS device, work has yet to be presented utilizing known concentrations of MeOH and EtOH-water mixtures and quantifying their effect on isotopic results returned by OA-ICOS analysis. The Liquid Water



Isotope Analyzer - Spectral Contamination Identifier (LWIA-SCI) software produced by LGR is used to identify and quantify the presence of spectral contaminants during liquid water analysis via OA-ICOS <sup>[4, 5]</sup>. Furthermore, if the contaminant sources are known, a protocol for correcting isotope ratios of contaminated samples has been suggested, though these protocols will be specific to individual devices <sup>[4]</sup>.

Here we repeat the case study of Brand *et al.* <sup>[1]</sup> but on the LGR OA-ICOS system. Where Brand *et al.* <sup>[1]</sup> compared the TC/EA-IRMS to a Picarro WS-CRDS system, we analyzed MeOH and EtOH contaminated samples on a LGR IWA-45EP OA-ICOS and an Elementar Isoprime IRMS system (Elementar UK Ltd, Stockport, UK). Since 2009, there has been an explosion of studies using the direct vapour equilibration (DVE) method <sup>[6-10]</sup>. However, for OA-ICOS measurements in vapour mode, by DVE or during in-situ analysis, no current contaminant detection or correction software exists. We go beyond the Brand *et al.* <sup>[1]</sup> case study, by also analyzing MeOH and EtOH contaminated samples in vapour mode via OA-ICOS. This is done to test the effectiveness of the LGR ‘Absorption’ (Ab) plot, and the ‘Spectra Fit Residuals’ plot (SFR), at indicating the presence of contaminants in analyzed samples during vapour analysis. We test the null hypothesis that results from the Brand *et al.* <sup>[1]</sup> study will be similar on the OA-ICOS system. We seek to reject the null hypothesis based on the findings of West *et al.* <sup>[2]</sup> where they showed significant differences in returned water isotopic composition between OA-ICOS and CRDS when analyzing organic contaminated samples. Our specific objectives were:

1. Quantify the effects of increasing concentrations of MeOH and EtOH contamination on OA-ICOS analysis in liquid and vapour analysis modes.
2. Investigate the effectiveness of the Ab and SFR plots at indicating the presence of MeOH and EtOH contaminants in samples analysed by OA-ICOS in vapour-mode.

### **4.3 Materials and Methods**

We prepared a series of pure and contaminated water samples using MeOH and EtOH as our contaminant source. Samples were prepared in 250-mL Nalgene™ HDPE bottles, with a new bottle used for each contamination concentration and type of contaminant (MeOH and EtOH).

The MeOH source was EMD Chemicals (Millipore Sigma, Burlington, MA, USA), histology grade, 98% by volume methanol. The EtOH source was Commercial Alcohols (Greenfield Global Inc, Toronto, ON, Canada), Ethyl Alcohol 95% by volume. The dilution equation ( $C_1V_1 = C_2V_2$ ) was used to determine appropriate volumes of contaminant per desired concentration level. Appropriate volumes of contaminant were mixed with 250 ml of deionized water (DIw) to create MeOH-water and EtOH-water mixtures. MeOH contaminated samples were prepared at 0.001%, 0.01%, 0.1%, 0.5%, and 1.0% concentration. EtOH contaminated samples were prepared at 0.001%, 0.01%, 0.1%, 1.0%, 5.0%, and 10.0% concentration. Sub samples were taken from the original MeOH-water and EtOH-water mixtures for liquid and vapour-mode analysis on OA-ICOS, and for liquid analysis on IRMS. For liquid water analysis, a series of sub-samples were prepared in replicate for the DIw control samples, and for each contamination concentration. In liquid-mode analysis, for the two isotopic analysis systems tested (OA-ICOS and IRMS), three replicates were created for each concentration and for the control DIw samples. For vapour-mode analysis by DVE on OA-ICOS, one sample was created per contaminant concentration. Thus, for liquid-mode analysis on OA-ICOS and IRMS, 36 samples were analysed: 3 DIw control samples, 15 MeOH contaminated water samples, and 18 EtOH contaminated water samples per analysis approach. For vapour-mode analysis on OA-ICOS 5 MeOH contaminated water samples and 6 EtOH contaminated water samples were analysed.

#### 4.3.1 Isotopic Analyses

Liquid water isotopic analysis of the control and contaminated samples was carried out on a LGR IWA-45EP OA-ICOS system and an Elementar Isoprime IRMS system. The DVE method was used to analyse contaminated samples in vapour-mode on only the OA-ICOS system.

For IRIS analyses in liquid-mode, an LGR IWA-45EP OA-ICOS analyser was used with an accuracy of  $\pm 1.0\text{‰}$  for  $\delta^2\text{H}$  and  $\pm 0.2\text{‰}$  for  $\delta^{18}\text{O}$  values. In vapour mode, for a 30 second reading period, results were accurate to  $\pm 1.8$  for  $\delta^2\text{H}$  and  $\pm 0.3$  for  $\delta^{18}\text{O}$  values. The liquid isotopic data were checked for spectral contamination using the Spectral Contamination Identifier (LWIA-SCI) post-processing software (LGR). For vapour-mode analysis by DVE we followed protocols developed by Wassenaar *et al.* <sup>[7]</sup>. For DVE analysis, one sample was prepared per contaminant

concentration by placing 5 ml of the sample into a 17.8 cm x 20.3 cm Leakproof bag (Uline, Wisconsin, USA. Model No. S-5855) with a double-locking airtight zipper. The headspace was filled with dry air and allowed to equilibrate with the samples for 30 minutes at room temperature ( $\sim 22^\circ\text{C}$ ) prior to analysis. The headspace sampling apparatus was a 21 G stainless steel needle connected to a 1 m long by 0.95 mm impermeable plastic line that was attached to the input port of the LGR analyser. Prior to, and in between sampling of headspaces, the needle was connected to a Drierite laboratory gas drying unit (W.A. Hammond Drierite Co. Ltd, Xenia, OH, USA) until the internal water content of the IWA-45EP analyser was below 500 ppmV  $\text{H}_2\text{O}$ . The headspace of control, contaminant-water, and water standard bags were sampled by piercing the sample bag with the needle. Sampling took on average two minutes. Water standard bags were re-sealed with tape in between analyses.  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  values were noted when the measured headspace water content stabilized at  $\sim 28,000$  ppmV  $\text{H}_2\text{O}$  for at least one minute and the standard deviation of the raw isotope data was less than  $\pm 1$  for both isotopes. Two lab water standards were used to normalize the data using the bracketed normalization configuration, which minimizes and controls for machine drift. Standard one had isotopic composition  $-201\text{‰}$  ( $\delta^2\text{H}$ ) and  $-26.17\text{‰}$  ( $\delta^{18}\text{O}$ ). Standard two had isotopic composition  $2.5\text{‰}$  ( $\delta^2\text{H}$ ) and  $-0.1\text{‰}$  ( $\delta^{18}\text{O}$ ). Control water samples were prepared in the same fashion as the contaminated samples, noted above. Water standards were alternated and analysed every four samples (bracketed normalization). A third, known, control water sample was analysed every eight samples as a means of tracking the quality assurance/ control of the run. The control water isotopic composition was,  $-137.5\text{‰}$  ( $\delta^2\text{H}$ ) and  $-17.45\text{‰}$  ( $\delta^{18}\text{O}$ ).

For IRMS analyses, an Elementar Isoprime isotope ratio mass spectrometer (Elementar UK Ltd, Stockport, UK) was used. The hydrogen isotopic compositions were determined by reduction of water to hydrogen by reaction with elemental chromium, following the method of Morrison *et al.* [11]. System protocols were as follows:  $0.8\ \mu\text{L}$  of water was injected into a quartz reactor containing elemental chromium at  $1030^\circ\text{C}$ . The resultant  $\text{H}_2$  gas was separated on a 1 mole sieve gas chromatograph column and introduced into the Elementar Isoprime isotope ratio mass spectrometer. Resultant raw delta values of the measured hydrogen were normalized to the VSMOW-SLAP scale by analyses of two calibrated waters, INV1 and ROD3, with  $\delta^2\text{H}$  values of

-218‰ and -4‰, respectively. For oxygen isotopes, we used the CO<sub>2</sub>-H<sub>2</sub>O equilibration technique of Epstein and Mayeda <sup>[12]</sup>. A Micromass multi-flow device (Waters Corp., Milford, MA, USA) was connected to the Elementar Isoprime isotope ratio mass spectrometer at 25 °C for all CO<sub>2</sub>-H<sub>2</sub>O equilibrations. Results are reported relative to the VSMOW-SLAP scale by normalizing to INV1 and ROD3, with δ<sup>18</sup>O = -28.5‰ and -1.0‰, respectively. The mass spectrometer used in this study had a precision of ±1.5‰ for δ<sup>2</sup>H and ±0.3‰ for δ<sup>18</sup>O values.

The isotope ratios (R) for δ<sup>2</sup>H (<sup>2</sup>H/<sup>1</sup>H) and δ<sup>18</sup>O (<sup>18</sup>O/<sup>16</sup>O) are expressed in per mil (‰) relative to Vienna Standard Mean Ocean Water (VSMOW), defined as follows:

$$\delta^2\text{H or } \delta^{18}\text{O} = (R_{\text{SAMPLE}} / R_{\text{STANDARD}} - 1) \quad (1)$$

Where R<sub>SAMPLE</sub> is the <sup>2</sup>H/<sup>1</sup>H and <sup>18</sup>O/<sup>16</sup>O ratios of the sample. R<sub>STANDARD</sub> is the <sup>2</sup>H/<sup>1</sup>H and <sup>18</sup>O/<sup>16</sup>O ratios of the VSMOW standard <sup>[13, 14]</sup>.

#### *4.3.2 Use of Absorption and Spectra Fit Residuals Plots for Contamination Detection*

The OA-ICOS system has two plots available for viewing in real time during vapour-mode analysis, that may be useful tools for indicating the presence of organic contaminants. They are the ‘Absorption’ (Ab) plot, and the ‘Spectra Fit Residuals’ (SFR) plot. During analysis of pure water samples both plots show consistent, stable absorption peaks at 5, 0, and -5 GHz, with smooth troughs between each peak with absorption plot values < 0.1 (Fig 1). In previous testing, during analysis of known contaminated samples, we noted that these smooth troughs began to fluctuate, indicating that these plots could be useful contamination detection tools. During DVE analysis in vapour-mode we monitor both Ab and SFR plots in real time during control and contaminated sample analysis, using fluctuations in the troughs to indicate contamination.

#### *4.3.3 Data Analysis*

Analysis was carried out with the statistical software R 3.3.2. <sup>[15]</sup> For liquid water analysis, to quantify consistency and repeatability, mean and standard deviation (sd) were calculated for the replicates of the control and contaminated samples for both analysis types. The stable isotope

ratios of the control DIw, analysed via OA-ICOS was considered the reference control water stable isotope values. We use the OA-ICOS result as our reference value as the accuracy of the OA-ICOS system is better than that of the IRMS system used in this study. The  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  error (‰) (trueness) was calculated for all liquid and vapour OA-ICOS, and liquid IRMS control and contaminated samples as follows:

$$\delta^2\text{H or } \delta^{18}\text{O error (‰)} = R_{\text{Control}} - R_{\text{Measured}} \quad (2)$$

Where:  $R_{\text{Control}}$  is the mean  $\delta^2\text{H}$  or  $\delta^{18}\text{O}$  value (‰) of the control water samples as analysed on OA-ICOS.  $R_{\text{Measured}}$  is the mean measured  $\delta^2\text{H}$  or  $\delta^{18}\text{O}$  value (‰) of the pure water samples analysed via IRMS or the contaminated samples measured via IRMS or OA-ICOS (liquid and vapour).

For a quality threshold we adopted the maximum accepted bias (MAB) detailed in Martin-Gomez *et al.* [3]. The MAB is  $\pm 6\text{‰}$  ( $\delta^2\text{H}$ ) and  $\pm 0.8\text{‰}$  ( $\delta^{18}\text{O}$ ) and was set by the Isotope Hydrology Section of the International Atomic Energy Agency (IAEA) during a proficiency test for water isotope analysis [3]. While other studies suggest smaller ranges for accuracy analysis in hydrological research (ex: Wassenaar *et al.* [16]), we agreed with the approach of Martin-Gomez *et al.* [3] in using a larger MAB range since we are comparing results between different analysis methods.

## 4.4 Results

### 4.4.1 Effects of Contamination on Liquid Water Analysis.

Table 1 summarizes the descriptive statistics of the isotopic ratios of the liquid and vapour samples for the control DIw and for all MeOH and EtOH contaminated samples analysed on IRMS and OA-ICOS. Table 2 summarizes the  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  values error relative to the OA-ICOS measured reference water isotopic composition. The mean stable isotope ratios of the DIw control sample, analysed via OA-ICOS, were  $-60.02\text{‰}$  ( $\delta^2\text{H}$ ) (sd:  $0.41\text{‰}$ ,  $n=3$ ) and  $-8.50\text{‰}$  ( $\delta^{18}\text{O}$ ) (sd:  $0.09\text{‰}$ ,  $n=3$ ). The stable isotope ratios of the DIw control samples analysed via IRMS were similar to the OA-ICOS reference values. The mean difference between OA-ICOS and IRMS control DIw results were  $-1.14\text{‰}$  ( $\delta^2\text{H}$ ) and  $-0.02\text{‰}$  ( $\delta^{18}\text{O}$ ) (Table 2). For liquid water

samples analysed via OA-ICOS, the LWIA-SCI software flagged MeOH contaminated samples at 0.01%, 0.1%, 0.5%, and 1% for narrow band and broad band contamination. MeOH-0.01% samples were flagged only for narrow band contamination. The LWIA-SCI software flagged EtOH contaminated samples for broad band contamination only, at 1%, 5%, and 10%. MeOH and EtOH at all contamination concentrations did not have a large impact on the stable isotope results returned by IRMS, relative to the IRMS control DIw results (Fig 2). Conversely, MeOH and EtOH contamination had a substantial effect on results returned by OA-ICOS (Fig 3).

However, MeOH contamination had a far greater effect than EtOH on OA-ICOS analyses. For MeOH contamination at 0.001% and 0.01% the isotopic results produced by OA-ICOS were similar to the OA-ICOS DIw reference (control) stable isotope results, with the maximum difference being 2.8‰ for  $\delta^2\text{H}$  values and 1.6‰ for  $\delta^{18}\text{O}$  values (Table 2). For MeOH contamination at 0.1%, 0.5% and 1% the difference between the OA-ICOS results and OA-ICOS reference values became substantial with the maximum difference being 406‰ ( $\delta^2\text{H}$ ) and 261‰ ( $\delta^{18}\text{O}$ ). For EtOH contamination,  $\delta^2\text{H}$  results were not significantly affected when analysed on OA-ICOS until 10% EtOH concentration. OA-ICOS  $\delta^{18}\text{O}$  results were significantly affected at 5% and 10% EtOH concentrations. Of note, for results produced by OA-ICOS, with increasing MeOH contaminant concentration the relationship between  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  values is strongly linear ( $R^2 = 0.9993$ ) (Fig 3). Increasing EtOH contamination did not result in the same highly linear relationship between returned  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  values ( $R^2 = 0.5835$ ) (Fig 3).

Figures 4 and 5 shows the  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  error caused by MeOH and EtOH contamination for OA-ICOS and IRMS in liquid analysis modes. The  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  error for results from IRMS analysis were not consistent with increasing contaminant concentration (Table 2). For MeOH contamination the average  $\delta^2\text{H}$  error of IRMS results relative to the OA-ICOS reference value was -0.29‰ (sd: 0.88‰, n=15), with the smallest error from MeOH-0.5% at 0.14‰ and the largest error from MeOH-0.01% at -1.05‰. However, these all fall within the  $\delta^2\text{H}$  precision range of the IRMS ( $\pm 1.5\text{‰}$ ). The average  $\delta^{18}\text{O}$  error of IRMS results relative to the OA-ICOS reference value for MeOH contamination was -0.08‰ (sd: 0.48‰, n=15), with the smallest error from MeOH-0.1% at 0.09‰ and the largest error from MeOH-0.001% and MeOH-1.0% at

0.58‰ and -0.58‰ respectively. The largest  $\delta^{18}\text{O}$  error values do not fall within the IRMS  $\delta^{18}\text{O}$  precision range ( $\pm 0.3\text{‰}$ ). For EtOH contamination the average  $\delta^2\text{H}$  error of IRMS results relative to the OA-ICOS reference value was 0.14‰ (sd: 1.04‰, n=18), with the smallest error from EtOH-0.1% at 0.4‰ and the largest error from EtOH-10.0% at -1.08‰. Again, these values fall within the IRMS precision range. The average  $\delta^{18}\text{O}$  error of IRMS results relative to the OA-ICOS reference value for EtOH contamination was -0.91‰ (sd: 0.22‰, n=18), with the smallest error from EtOH-0.1% at -0.71‰ and the largest error from EtOH-10% at -1.11‰. Both the largest and smallest  $\delta^{18}\text{O}$  error values fell outside the IRMS precision range.

MeOH and EtOH contamination had a more significant effect on  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  error for liquid-mode results produced by OA-ICOS as shown in Figure 4 and 5 and in Table 2. MeOH contamination caused  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  values to become increasingly more positive as the concentration of contaminant increased (Fig 3). For MeOH contamination, the smallest  $\delta^2\text{H}$  error of OA-ICOS results relative to the OA-ICOS reference value was from MeOH-0.001% at 0.03‰ and the largest  $\delta^2\text{H}$  error was from MeOH-1.0% at 406.3‰. The smallest  $\delta^{18}\text{O}$  error of OA-ICOS results relative to the OA-ICOS reference value was MeOH-0.001% at -0.08‰ and the largest error from MeOH-1% at 261‰. For the lower concentrations (0.001%, 0.01%, 0.1%, 1.0%), EtOH contamination inconsistently affected the  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  values returned by OA-ICOS. At higher EtOH concentrations (5% and 10%) the  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  values skewed in a negative direction. For EtOH contamination the average  $\delta^2\text{H}$  error of OA-ICOS results relative to the OA-ICOS reference value was -2.83‰ (sd: 3.5‰, n=18), with the smallest error from EtOH-0.1% at -0.79‰ and the largest error from EtOH-10% at -9.84‰. The average  $\delta^{18}\text{O}$  error of OA-ICOS results for EtOH contamination was -1.7‰ (sd: 1.4‰, n=18), with the smallest error from EtOH-0.1% at -0.64‰ and the largest error from EtOH-10% at -4.1‰.

Vapour-mode results from OA-ICOS were even more significantly affected by MeOH and EtOH contamination than liquid-mode results (Figure 4 and 5, Table 2). Specifically, in liquid-mode analysis it was the  $\delta^2\text{H}$  results that were most strongly affected by the presence of contaminants, whereas in vapour-mode, the  $\delta^{18}\text{O}$  results were the far more strongly affected of the two isotopic ratios. For MeOH contamination the smallest  $\delta^2\text{H}$  error of OA-ICOS-vapour results relative to

the OA-ICOS reference value was from MeOH-0.001% at 2.51‰ and the largest measured error was from MeOH-0.5% at 941.7‰. The smallest  $\delta^{18}\text{O}$  error of OA-ICOS-vapour results relative to the OA-ICOS reference value for MeOH contamination was again from MeOH-0.001% at 1.08‰, and the largest measured error was from MeOH-0.5% at 1315.2‰. We note that isotopic values ( $\delta^2\text{H}$  and  $\delta^{18}\text{O}$ ) for MeOH-1% when measured in vapour-mode were so affected by contamination that the OA-ICOS system could not return a result (Table 2 NA values). For EtOH contamination the smallest  $\delta^2\text{H}$  error of OA-ICOS-vapour results relative to the OA-ICOS reference value was from EtOH-0.001% at -0.45‰, and the largest was from EtOH-5.0% at 33.1‰. The smallest  $\delta^{18}\text{O}$  error of OA-ICOS-vapour results relative to the OA-ICOS reference value for EtOH contamination was again from EtOH-0.001% at -0.5‰, and the largest error was from EtOH-0.5% at -43.4‰.

Figure 6 shows the MeOH-water and EtOH-water mixture results from IRMS and OA-ICOS (liquid-mode), detailing the offsets of the OA-ICOS results from the IRMS results for each contaminant, at each concentration level. The dependence of the results on MeOH contamination is clearly far greater than for EtOH contamination. The results from OA-ICOS liquid-mode analysis for both contaminants (MeOH and EtOH) show highly linear relationships between MeOH and EtOH contaminant concentrations and both isotope ratios ( $\delta^2\text{H}$  and  $\delta^{18}\text{O}$ ).

#### *4.4.2 Ability of Absorption and Spectral Fit Residual Plots to Detect Contaminants.*

Pure water, when analysed by DVE in vapour mode on OA-ICOS will yield Ab and SFR plots as shown in Figure 1, and the peaks (at 5, 0, and -5 Ghz) and troughs of these plots will remain in a consistent location with no fluctuations. When MeOH-water mixtures were analysed by DVE on the OA-IOCS in vapour-mode, the following was observed. At MeOH concentration of 0.001% neither the Ab or SFR plot showed any fluctuations (Fig 7A). At MeOH concentration 0.01% the Ab plot shows no evidence of fluctuation, but the SFR plot began to show fluctuations between the normal peaks (Fig 7B). At MeOH 0.1%, small fluctuations begin to appear in the troughs of the Ab plot, and the SFR plot shows substantial fluctuations (Fig 7C). Furthermore, at this concentration (MeOH 0.1%) the returned stable isotope results begin to clearly diverge from the DIw control results produced by OA-ICOS during DVE analysis. At MeOH 0.5% and 1.0%



both the Ab and SFR plots show large fluctuations in the troughs between absorption peaks, and in fact begin to show negative absorption values on the AB plot (Fig 7D and E). Since negative absorbance is a physical impossibility, this indicates that the results produced with negative absorbance should be discarded.

When EtOH-water mixtures were analysed by DVE via the OA-ICOS in vapour-mode, the following was observed. At EtOH concentrations of 0.001%, 0.01%, and 0.1% neither the Ab or SFR plots show any fluctuations (Fig 8A, B, and C). The returned stable isotope values at 0.001% and 0.01 % EtOH do not differ from the DIw control value returned by OA-ICOS DVE vapour-mode analysis. At 0.1% EtOH concentration, returned stable isotope results begin to diverge from control  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  values, though they remain within reasonable error ranges. At EtOH 1.0%, the AB plot still shows no signs of fluctuations, however the SFR plot line begins to become very noisy (Fig 8D) and the stable isotope results also plot further away from the DIw control stable isotope values. At EtOH 5% the Ab plot begins to show noise in the troughs between peaks and the SRF plot becomes very noisy (Fig 8E). Additionally, the stable isotope results also become substantially different from the DIw control. Finally, at EtOH 10% concentration the entire Ab plot line begins to fluctuate with noise and the SFR plot becomes so noisy that the peaks and troughs are eliminated (Fig 8F).

## 4.5 Discussion

### 4.5.1 Our Findings in the Context of Previous Work.

Our findings are consistent with Brand *et al.* <sup>[1]</sup>, West *et al.* <sup>[2]</sup>, and Martin-Gomez *et al.* <sup>[3]</sup> where the apparent effect of MeOH contamination was far more pronounced than the apparent effect of EtOH on returned IRIS results. We now confirm this finding for the LGR OA-ICOS system. One notable difference in our findings is that during liquid-mode analysis, MeOH contamination caused OA-ICOS isotopic results to skew positively and EtOH contamination caused returned isotopic results to skew negatively (Figure 3), but in West *et al.* <sup>[2]</sup> LGR OA-ICOS results tended to skew positively in the presence of contaminated samples. For the results that did skew negatively in the West *et al.* <sup>[2]</sup> study, they fell within the minimum instrument error (precision) range. Our findings suggest that EtOH contamination caused OA-ICOS results to skew in a

negative direction when concentrations were high enough to impact isotopic results. This was observed to some extent in Martin-Gomez *et al.* [3] for  $\delta^2\text{H}$  error on the CRDS system, but less so for  $\delta^{18}\text{O}$  error related to EtOH contamination. When West *et al.* [2] showed that LGR OA-ICOS results skewed positively in the presence of organic contaminated plant water, we postulate that their organic contaminated water contained both MeOH and EtOH (and likely other plant organic compounds) in varying concentrations. Since MeOH contamination produces a greater effect on returned isotopic results, any influence from EtOH contamination in the West *et al.* [2] plant water samples would be overpowered by MeOH effects. In the cases where the West *et al.* [2] LGR results skewed negatively, our EtOH results suggest that those plant water samples had a little to no MeOH contamination and were mostly affected by EtOH contamination. Part of the Martin-Gomez *et al.* [3] study detailed the effects of MeOH and EtOH-water mixtures on Picarro L2120-i and L1102-i CRDS isotopic analysers. They showed that EtOH contamination caused  $\delta^2\text{H}$  error to skew in a negative direction (as seen in our results) but that  $\delta^{18}\text{O}$  error skewed in a positive direction for the same EtOH contamination (not seen in our results). We note that for MeOH contamination at 1.0%, our OA-ICOS results were far more affected than the MeOH-1.0% WS-CRDS results of Brand *et al.* [1]. We agree with West *et al.* (2010) that device specific effects between LGR OA-ICOS and Picarro CRDS that are causing differences in the trueness (relative error) of results from contaminated samples is highly problematic. The differences between IRIS manufacturer's response to contaminants are likely related to the specific range of the water absorption band of the electromagnetic (EM) spectrum being utilized by each manufacturer. Additionally, the mathematical fitting procedures translating absorption into stable isotope values are likely unique to each manufacturer. As both the specific range of the EM water absorption band and mathematical fitting models utilized by Picarro and LGR are proprietary information, research into how these instrument specific feature affect returned IRIS results is difficult.

Even more problematic is the finding in our study that MeOH and EtOH contamination effects are not consistent between liquid-mode and vapour-mode analysis on OA-ICOS. In liquid-mode analysis MeOH contamination caused  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  results to skew positively, with the greater effect occurring to  $\delta^2\text{H}$  results. However, in vapour-mode, while  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  results still

skewed positively from MeOH contamination, the effect of MeOH contamination was far more powerful for vapour-mode results, and the greater effect was on  $\delta^{18}\text{O}$  results instead. (Table 2, Figure 4 and 5). Indeed, for MeOH-0.5%, vapour-mode results were 769‰ ( $\delta^2\text{H}$ ) and 1197‰ ( $\delta^{18}\text{O}$ ) more positive than liquid-mode results for the same contaminant concentration. In liquid-mode analysis EtOH contamination caused both  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  OA-ICOS results to skew negatively, though its effect was far smaller than MeOH. Interestingly, in vapour-mode, EtOH caused  $\delta^2\text{H}$  error to skew positively, but  $\delta^{18}\text{O}$  error to skew negatively. We suggest that the increased effect of MeOH and EtOH contamination on vapour-mode analysis results is related to the relative volatility of MeOH and EtOH. Both MeOH and EtOH have higher volatility (lower vaporization temperature) than water. As such, even though DVE analysis is taking place at room temperature, it may be possible that larger fractions of MeOH and EtOH are moving into vapour phase relative to the amount of water also moving into vapour phase. Thus, the concentrations of MeOH and EtOH may be higher in the headspace of the sample than is originally prepared in the liquid sample. If this is the case, then plant samples analysed in vapour mode (by DVE) that have a potential for organic contamination should be closely monitored with available contaminant detection tools. As this toolkit is currently quite limited, detection techniques for use during vapour-mode analysis must be advanced. We discuss this further, below.

For MeOH contamination we found +406‰ relative error for  $\delta^2\text{H}$  and +261‰ relative error for  $\delta^{18}\text{O}$  (relative to our OA-ICOS reference values). This was much greater than errors found by Brand *et al.* (2009) where they reported  $\sim -23\%$  relative error for  $\delta^2\text{H}$  and  $\sim -11\%$  relative error for  $\delta^{18}\text{O}$ . However, our EtOH results, shown in Figure 6, resemble the results of the Brand *et al.* study, in that our OA-ICOS results for both isotope ratios ( $\delta^2\text{H}$  and  $\delta^{18}\text{O}$ ) skewed negatively with increasing contamination. This was unexpected as we originally assumed that both EtOH and MeOH contamination would cause a positive skew in  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  results from OA-ICOS based on observations by West *et al.* [2]. While Martin-Gomez *et al.* [3] showed a negative skew in the trueness of  $\delta^2\text{H}$  results for EtOH contaminated samples, we hypothesized that this was a device-specific effect on trueness unique to the CRDS system. We observed a linear correlation between MeOH concentrations and both stable isotope ratios, and EtOH concentrations and both stable

isotope ratios, as was reported by Brand *et al.* <sup>[1]</sup> (Figure 6). But, our linear relationships between contaminant concentrations and stable isotope ratios were not identical to those of Brand *et al.* <sup>[1]</sup>. This may be due to the unique stable isotope composition ( $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  ratios) of our MeOH and EtOH sources relative to those used in Brand *et al.* <sup>[1]</sup>. We too attribute the effects of contamination on returned OA-ICOS results to be feature specific spectral contamination effects, as opposed to mass-based fractionation effects.

The findings of our research and Martin-Gomez *et al.* <sup>[3]</sup> while similar in that MeOH contamination caused far greater effects than EtOH contamination for IRIS our analyses are different in that we showed opposite effects on trueness skew direction (positive vs negative) from organic contamination. For IRIS results, Martin-Gomez *et al.* <sup>[3]</sup> showed that EtOH contamination caused the CRDS  $\delta^{18}\text{O}$  results to skew positively, whereas our OA-ICOS  $\delta^{18}\text{O}$  result trueness skewed in the negative direction (Fig 5). The  $\delta^{18}\text{O}$  trueness results from Martin-Gomez *et al.* <sup>[3]</sup> fell within the MAB. In our study  $\delta^{18}\text{O}$  trueness results fell within the MAB, except for the EtOH-1.0%, 5.0%, and 10.0% results. For EtOH contamination effects on  $\delta^2\text{H}$  trueness, we found only minor effects with a mix of positive and negative trueness skew. EtOH contamination caused  $\delta^2\text{H}$  errors that fell well within the MAB, except for results from EtOH-10.0% concentration. However, Martin-Gomez *et al.* <sup>[3]</sup> found trueness skew in  $\delta^2\text{H}$  results from EtOH contamination at 2%, 4% and 8% concentrations. For EtOH at 8%, the Martin-Gomez *et al.* <sup>[3]</sup>  $\delta^2\text{H}$  results fell outside of the MAB.

The effect of MeOH contamination on  $\delta^{18}\text{O}$  trueness on IRIS results in our study (via OA-ICOS) was substantially larger than that of Martin-Gomez *et al.* <sup>[3]</sup> (via CRDS). MeOH contamination caused our  $\delta^{18}\text{O}$  trueness to skew positively, whereas it caused the  $\delta^{18}\text{O}$  trueness of Martin-Gomez *et al.* <sup>[3]</sup> to skew negatively. Our maximum trueness skew for  $\delta^{18}\text{O}$  was 261‰ relative to our OA-ICOS reference value (-8.5‰), occurring at MeOH-1.0%, whereas the maximum  $\delta^{18}\text{O}$  trueness skew from Martin-Gomez *et al.* <sup>[3]</sup> was -142‰ from MeOH-1.6%. Conversely, the effect of MeOH contamination on  $\delta^2\text{H}$  error was far more substantial in the Martin-Gomez *et al.* <sup>[3]</sup> study than was the case in our experiment. We found a maximum  $\delta^2\text{H}$  trueness skew of +406‰ at MeOH-1.0% relative to the OA-ICOS reference value (-60.02‰) (Fig 6). Martin-

Gomez *et al.* <sup>[3]</sup> found a maximum  $\delta^2\text{H}$  error of -1077‰ relative to their control. While the Martin-Gomez *et al.* <sup>[3]</sup> maximum MeOH concentration was almost double our values (1.6% vs 1.0%, respectively), our use of the linear relationship between  $\delta^2\text{H}$  results and MeOH contaminant concentration ( $\delta^2\text{H}$  ratio =  $400.16 \times (\text{MeOH [1.6\%]}) - 65.57$ ,  $R^2 = 0.994$ ) (Fig 6) for MeOH at 1.6% concentration, results in a  $\delta^2\text{H}$  ratio of 575‰, which is a relative error of 636‰. So, our maximum  $\delta^2\text{H}$  error was still lower than that found by Martin-Gomez *et al.* <sup>[3]</sup>.

For IRMS analyses, our study and that of Martin-Gomez *et al.* <sup>[3]</sup> found that EtOH and MeOH contamination had very little effect on returned IRMS results. However, Martin-Gomez *et al.* <sup>[3]</sup> showed that for EtOH-8%  $\delta^{18}\text{O}$  error fell outside of the MAB, and for EtOH-4% and 8%  $\delta^2\text{H}$  error fell outside of the MAB. For all MeOH concentrations and for both isotopes none of Martin-Gomez *et al.* <sup>[3]</sup> results fell outside of the MAB. In our study the IRMS  $\delta^{18}\text{O}$  error fell outside of the MAB at EtOH 1%, 5%, and 10%. For  $\delta^2\text{H}$  error, EtOH stable isotope results at all concentrations did not fall outside of the MAB. Similarly, for both isotope ratios ( $\delta^2\text{H}$  and  $\delta^{18}\text{O}$ ), MeOH contamination at all concentrations did not cause IRMS results to fall outside of the MAB. The differences between the results of our study and the findings of other researchers <sup>[1-3]</sup> is likely connected to device specific measurement differences, and the unique isotopic compositions of the contaminants chosen in the various studies.

#### 4.5.2 Absorption and Spectral Fit Residuals Plots as Contaminant Detectors.

Millar *et al.* <sup>[17]</sup> showed that DVE may be an effective method for investigating the stable isotope ratios of plant transpiration stream water. However, an effective means of gauging the presence of organic contaminants in the headspace of DVE samples is urgently needed. As shown in the results section above, the Ab plot appears to only be useful as a MeOH contaminant detection tool during vapour-mode analysis of DVE samples on OA-ICOS at MeOH concentrations  $\geq 0.1\%$  (Fig 7). The Ab plot is even less effective at detecting EtOH contamination, as it only begins to show evidence of contamination at concentrations  $\geq 5.0\%$  (Fig 8). The SFR plot is much more effective than the Ab plot at detecting MeOH contamination in the headspace of DVE samples at concentrations as low as 0.01%, which is when stable isotope results begin to substantially diverge from the OA-ICOS reference values. The SFR plot is also more effective at

detecting EtOH contamination than the Ab plot, with evidence of contamination observable at concentrations as low as 0.1%, which is again when stable isotope results begin to diverge from the OA-ICOS reference values. Since MeOH is the more problematic of the two compounds, causing greater effects on returned stable isotope results as shown in our study and others <sup>[1, 3]</sup>, it's detection and correction should be prioritized. As such, the SFR plot appears to be an effective tool for detection. The offsets displayed on the SFR plot in the presence of contaminants are based on output parameters in the raw data files of the OA-ICOS system. These offsets are correlated with the organic contaminants present in the measurement cavity. Thus, it should be possible to correct isotopic data and possibly quantify the concentration of contaminants based on these raw output parameters. We suggest this may be possible based on similar work utilizing the LWIA-SCI (LGR) software <sup>[4]</sup>. In this work Schultz *et al.* <sup>[4]</sup> show that the correction approach they developed was able to eliminate differences between IRMS and IRIS results for  $\delta^{18}\text{O}$ , and substantially reduce differences for  $\delta^2\text{H}$ . However, they noted differences in the magnitude and direction of corrections between their developed approach and that detailed by LGR. As such they note that unique correction protocols will likely need to be developed for individual analysis systems. This work suggest that correction factors developed for vapour analysis approaches will also need to be device specific.

#### 4.6 Conclusion

We analysed a series of MeOH-water and EtOH-water mixtures of varying concentrations on IRMS and LGR OA-ICOS analysis systems. We report similar findings to that of previous work with the Picarro CRDS analyser whereby MeOH and EtOH contamination cause errors in returned stable isotope results. Our work confirms that MeOH is a far more problematic contaminant than EtOH, causing larger relative errors in  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  ratios returned by OA-ICOS analysis. While our findings are similar to previous work with the Picarro device, the magnitude of errors, the direction of relative  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  trueness skew (positive vs negative), and the offsets between control values, and contaminated sample OA-ICOS results were not. This is likely due to a combination of factors including the use of different IRIS methods (OA-ICOS vs WS-CRDS) and the difference in stable isotope composition of the MeOH and EtOH

used as contaminant sources in the various studies, and device and manufacturer specific differences.

While software like the LWIA-SCI package exists for organic contaminant detection during liquid water analysis, no such systems exist for use during vapour-mode analysis on OA-ICOS systems. We found that the Spectral Fit Residuals plot, available on LGR OA-ICOS systems, may be a useful tool for organic contaminant detection in real time at concentrations where results begin show evidence of errors, and thus that correction based on raw data output parameters could be developed. The SFR plot could be very useful during DVE analysis for flagging samples that are contaminated and in need of correction or analysis via IRMS. We advocate that further tools be developed for contaminant detection, in-line filtration, and isotopic result correction during vapour-mode analysis approaches.

#### **4.7 Acknowledgments**

Thanks are due to Dr. Bingchen Si, Dr. Marcel Gaj, and the McDonnell Watershed Hydrology lab group. This research was supported by an NSERC Discovery grant to JJM, and by the NSERC CREATE program.

#### **4.8 Transition Statement**

In Chapter 4 we quantified the effects of methanol and ethanol contamination of the LGR OA-ICOS analysis approach. Our findings agree with previous work in that MeOH causes significantly more of an effect on isotopic results than EtOH. Not seen before is the finding that MeOH contamination and EtOH contamination cause OA-ICOS result error to skew in opposite directions. Chapter 4 is the final scientific study of this thesis. Chapter 5 will contain concluding remarks, study limitations and future research recommendations.

#### **4.9 Author Contributions**

C. Millar designed the experiment, prepared the samples, analysed the data, and wrote the manuscript. K. Janzen carried out liquid water and vapour analysis of the samples via OA-ICOS and IRMS; prepared a report on the Ab and SFR plot contaminant detection capabilities that was

the basis for portions of the manuscript; and edited the manuscript. J.J. McDonnell, D. Schneider, and G. Koehler edited the manuscript and provided critical feedback and insights into understanding results and assisted in development of the discussion section of the manuscript.

#### 4.10 References

- [1] Brand W A, Geilmann H, Crosson E R, Rella C W. Cavity ring-down spectroscopy versus high-temperature conversion isotope ratio mass spectrometry; a case study on  $\delta(2)\text{H}$  and  $\delta(18)\text{O}$  of pure water samples and alcohol/water mixtures. *Rapid Commun Mass Sp* 2009; 23(12):1879-1884.
- [2] West A G, Goldsmith G R, Brooks P D, Dawson T E. Discrepancies between isotope ratio infrared spectroscopy and isotope ratio mass spectrometry for the stable isotope analysis of plant and soil waters. *Rapid Commun Mass Sp* 2010; 24(14):1948-1954.
- [3] Martin-Gomez P, Barbeta A, Voltas J, Penuelas J, Dennis K, Palacio S, Dawson T E, Ferrio J P. Isotope-ratio infrared spectroscopy: A reliable tool for the investigation of plant-water sources? *New Phytol* 2015; 207(3):914-927.
- [4] Schultz N M, Griffis T J, Lee X, Baker J M. Identification and correction of spectral contamination in  $2\text{H}/1\text{H}$  and  $18\text{O}/16\text{O}$  measured in leaf, stem, and soil water. *Rapid Commun Mass Spectrom* 2011; 25(21):3360-3368.
- [5] Leen J B, Berman E S F, Liebson L, Gupta M. Spectral contaminant identifier for off-axis integrated cavity output spectroscopy measurements of liquid water isotopes. *Rev Sci Instrum* 2012; 83(4)
- [6] Hendry M J, Schmeling E, Wassenaar L I, Barbour S L, Pratt D. Determining the stable isotope composition of pore water from saturated and unsaturated zone core: Improvements to the direct vapour equilibration laser spectrometry method. *Hydrol Earth Syst Sc* 2015; 19(11):4427-4440.
- [7] Wassenaar L I, Hendry M J, Chostner V L, G.P. L. High resolution pore water  $\delta 2\text{H}$  and  $\delta 18\text{O}$  measurements by  $\text{H}_2\text{O}(\text{liquid})\text{-H}_2\text{O}(\text{vapor})$  equilibration laser spectroscopy. *Envir Sci Tech* 2008; 42(24):9262-9267.
- [8] Pratt D L, Lu M, Barbour S L, Hendry M J. An evaluation of materials and methods for vapour measurement of the isotopic composition of pore water in deep, unsaturated zones. *Isotopes in Environmental and Health Studies* 2016; 52(4-5):529-543.
- [9] Wang L, Good S P, Caylor K K, Cernusak L A. Direct quantification of leaf transpiration isotopic composition. *Agricultural and Forest Meteorology* 2012; 154-155(127-135).
- [10] Song X, Barbour M M. Leaf water oxygen isotope measurement by direct equilibration. *New Phytol* 2016; 211(3):1120-1128.
- [11] Morrison J, Brockwell T, Merren T, Fourel F, Phillips A M. On-line high precision stable hydrogen isotopic analyses on nanoliter water samples. *Anal Chem* 2001; 73(15):3570–3575.
- [12] Epstein S, Mayeda T. Variation of  $\delta 18\text{O}$  content of waters from natural sources. *Geochim Cosmochim Ac* 1953; 4(5):213-224.
- [13] Craig H. Standard for reporting concentration of deuterium and oxygen-18 in natural waters. *Science* 1961; 133(3467):1833-1834.



- [14] Coplen T B. Guidelines and recommended terms for expression of stable-isotope-ratio and gas-ratio measurement results. *Rapid Commun Mass Sp* 2011; 25(17):2538-2560.
- [15] CoreTeam R, 3.3.2 ed., R Foundation for Statistical Computing, Vienna, Austria., **2013**.
- [16] Wassenaar L I, Ahmad M, Aggarwal P, van Duren M, Poltenstein L, Araguas L, Kurttas T. Worldwide proficiency test for routine analysis of delta2h and delta18o in water by isotope-ratio mass spectrometry and laser absorption spectroscopy. *Rapid Commun Mass Sp* 2012; 26(15):1641-1648.
- [17] Millar C, Pratt D, Schneider D J, McDonnell J J. A comparison of extraction systems for plant water stable isotope analysis. *Rapid Commun Mass Sp* 2018; 32(13):1031-1044.
- [18] Pham S V, Leavitt P R, McGowan S, Wissel B, Wassenaar L I. Spatial and temporal variability of prairie lake hydrology as revealed using stable isotopes of hydrogen and oxygen. *Limnol Oceanogr* 2009; 54(1):101-118.

#### 4.11 Tables and Figures

**Table 4.1.** Means and standard deviations (sd) of  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  ratios from control DIw samples and from MeOH-water and EtOH-water contaminated samples. The stable isotope ratios for DIw controls, analysed via OA-ICOS, are considered the reference (true) DIw value. Note: the vapour analysis samples for OA-ICOS had one data point each (not replicated at n=3 as in the liquid analysis for IRMS and OA-ICOS). NA indicates that no value was noted.

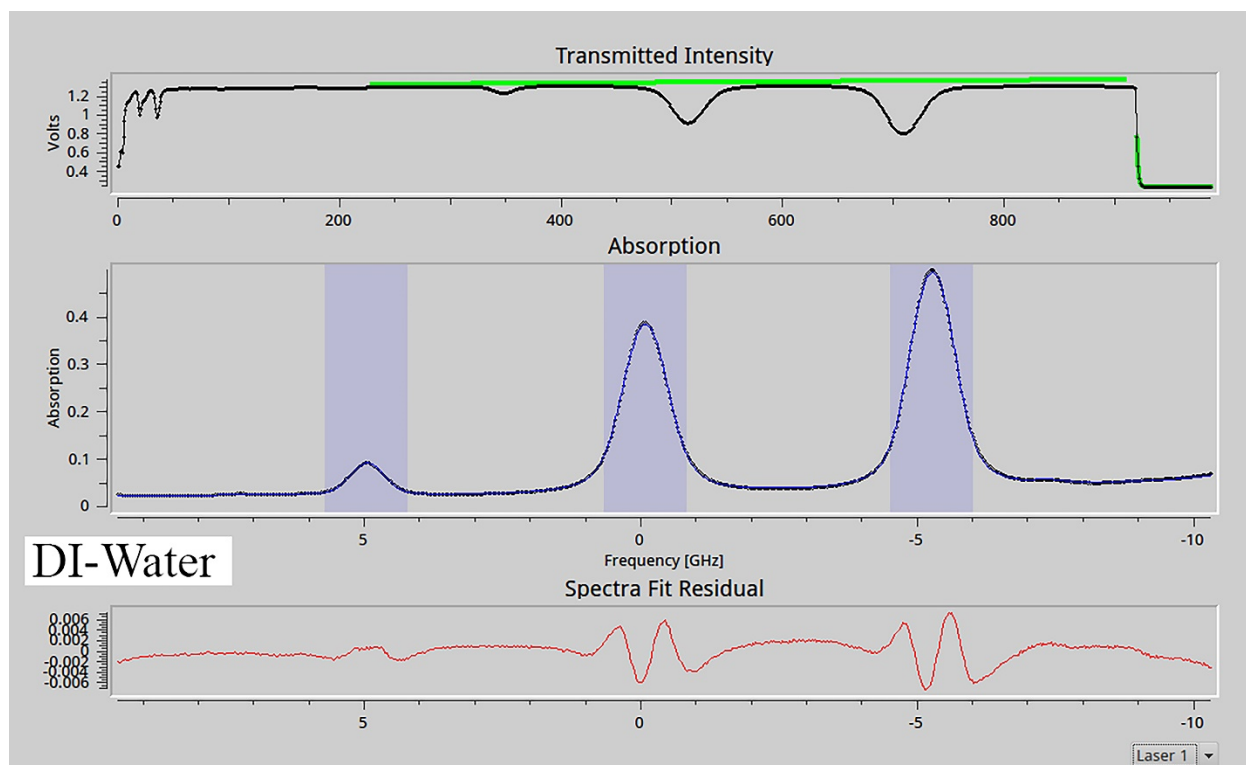
Sample ID	n	Results of Analysis on OA-ICOS						Results of Analysis on IRMS			
		Liquid Analysis				Vapour Analysis		Liquid Analysis			
		$\delta^2\text{H}$		$\delta^{18}\text{O}$		$\delta^2\text{H}$ (‰)	$\delta^{18}\text{O}$ (‰)	$\delta^2\text{H}$		$\delta^{18}\text{O}$	
		Average (‰)	sd (‰)	Average (‰)	sd (‰)			Average (‰)	sd (‰)	Average (‰)	sd (‰)
Control H <sub>2</sub> O	3	-60.02	0.41	-8.50	0.09	-62.09	-8.80	-61.16	0.06	-8.52	0.20
Methanol Contaminated Samples											
MeOH-0.001%	3	-59.99	0.17	-8.58	0.31	-57.51	-7.42	-60.49	1.13	-7.92	0.50
MeOH-0.01%		-57.23	0.20	-6.91	0.03	-32.93	7.00	-61.07	0.45	-8.60	0.20
MeOH-0.1%		-29.71	0.23	12.98	0.09	251.73	159.79	-60.28	0.60	-8.41	0.31
MeOH-0.5%		111.91	0.25	108.91	0.35	881.68	1306.70	-59.87	1.23	-8.87	0.19
MeOH-1.0%		346.26	1.03	252.54	0.43	NA	NA	-59.83	0.74	-9.08	0.04
Ethanol Contaminated Samples											
EtOH-0.001%	3	-62.43	1.22	-9.29	0.55	-60.47	-9.00	-59.05	0.33	-9.18	0.05
EtOH-0.01%		-60.90	0.13	-9.22	0.02	-62.67	-9.01	-59.40	1.01	-9.30	0.03
EtOH-0.1%		-60.81	0.36	-9.14	0.08	-54.42	-9.97	-59.61	1.19	-9.21	0.04
EtOH-1.0%		-62.03	0.28	-9.44	0.02	-51.61	-15.16	-59.27	0.54	-9.58	0.03
EtOH-5.0%		-61.06	0.43	-11.44	0.00	-26.90	-35.67	-60.81	0.17	-9.69	0.02
EtOH-10.0%		-69.86	0.09	-12.58	0.11	-48.55	-51.91	-61.09	0.88	-9.61	0.16

**Table 4.2.** Relative error (trueness) of  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  values measured by IRIS and OA-ICOS. The OA-ICOS DIw isotope ratios are used as the reference (true) control water isotope composition. Relative error is calculated as detailed in Equation 2 in the methods section. The precision of the OA-ICOS system in liquid analysis mode is:  $\pm 1.0\text{‰}$  for  $\delta^2\text{H}$  and  $\pm 0.2\text{‰}$  for  $\delta^{18}\text{O}$  values, and in vapour mode it is:  $\pm 1.8$  for  $\delta^2\text{H}$  and  $\pm 0.3$  for  $\delta^{18}\text{O}$  values. The precision of the IRMS system is  $\pm 1.5\text{‰}$  for  $\delta^2\text{H}$  and  $\pm 0.3\text{‰}$  for  $\delta^{18}\text{O}$  values.

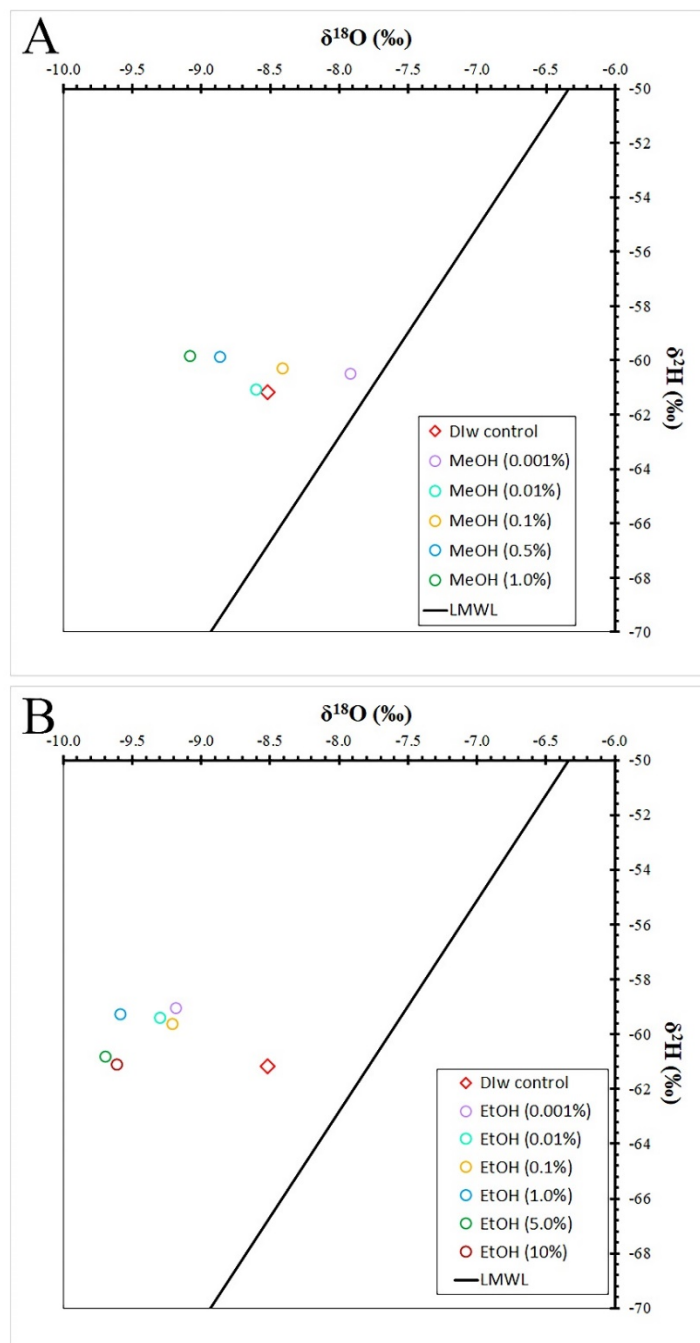
	$\delta^2\text{H}$ (‰)	$\delta^{18}\text{O}$ (‰)
OA-ICOS Control Reference Value	-60.02	-8.50

$\delta^2\text{H}$ and $\delta^{18}\text{O}$ error [‰] (trueness) relative to reference value.						
Sample ID	OA-ICOS				IRMS	
	Liquid Analysis		Vapour Analysis		Liquid Analysis	
	$\delta^2\text{H}$ error (‰)	$\delta^{18}\text{O}$ error (‰)	$\delta^2\text{H}$ error (‰)	$\delta^{18}\text{O}$ error (‰)	$\delta^2\text{H}$ error (‰)	$\delta^{18}\text{O}$ error (‰)
IRMS Control	-	-	-	-	-1.14	-0.02
MeOH-0.001%	0.03	-0.08	2.51	1.08	-0.47	0.58
MeOH-0.01%	2.79	1.60	27.09	15.50	-1.05	-0.10
MeOH-0.1%	30.31	21.48	311.75	168.29	-0.26	0.09
MeOH-0.5%	171.93	117.42	941.70	1315.20	0.14	-0.37
MeOH-1.0%	406.27	261.04	NA	NA	0.19	-0.58
EtOH-0.001%	-2.41	-0.79	-0.45	-0.50	0.97	-0.68
EtOH-0.01%	-0.88	-0.72	-2.65	-0.51	0.61	-0.80
EtOH-0.1%	-0.79	-0.64	5.60	-1.47	0.40	-0.71
EtOH-1.0%	-2.01	-0.93	8.41	-6.66	0.74	-1.08
EtOH-5.0%	-1.04	-2.94	33.12	-27.17	-0.79	-1.19
EtOH-10.0%	-9.84	-4.08	11.47	-43.41	-1.08	-1.11

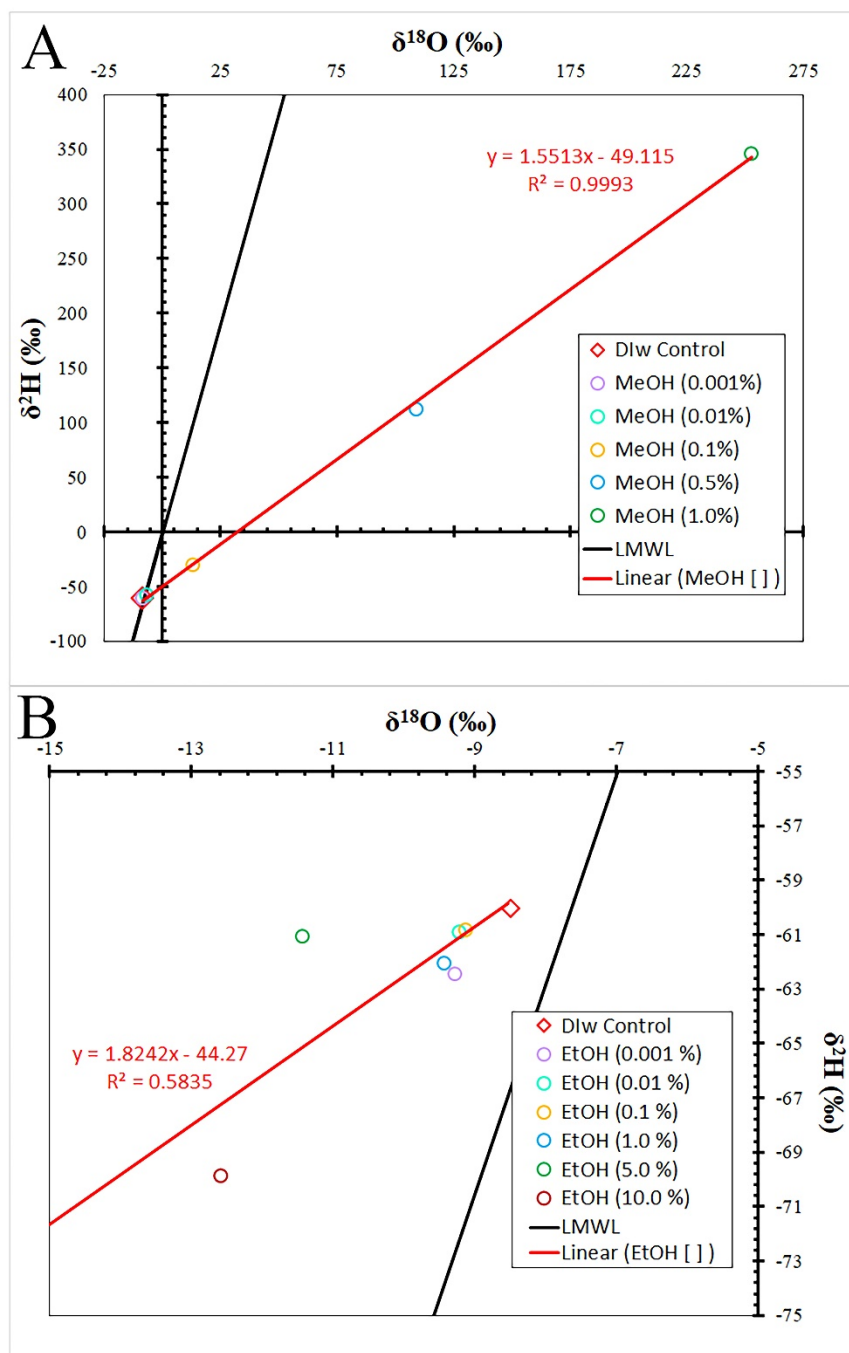
**Figure 4.1.** Screenshot of ‘Absorption’ plot (center graph) and ‘Spectra Fit Residual’ plot (lower graph) from LGR OA-ICOS analyser during analysis of de-ionized, (uncontaminated) water. On the Absorption plot, the peak at 5 GHz is for the water isotopologue  $^1\text{H}^2\text{HO}$ , the peak at 0 GHz is for  $\text{H}_2^{16}\text{O}$ , and the peak at -5 GHz is for  $\text{H}_2^{18}\text{O}$ . During analysis of an uncontaminated samples, the peaks in both plots are stable and consistently remain at the noted frequencies.



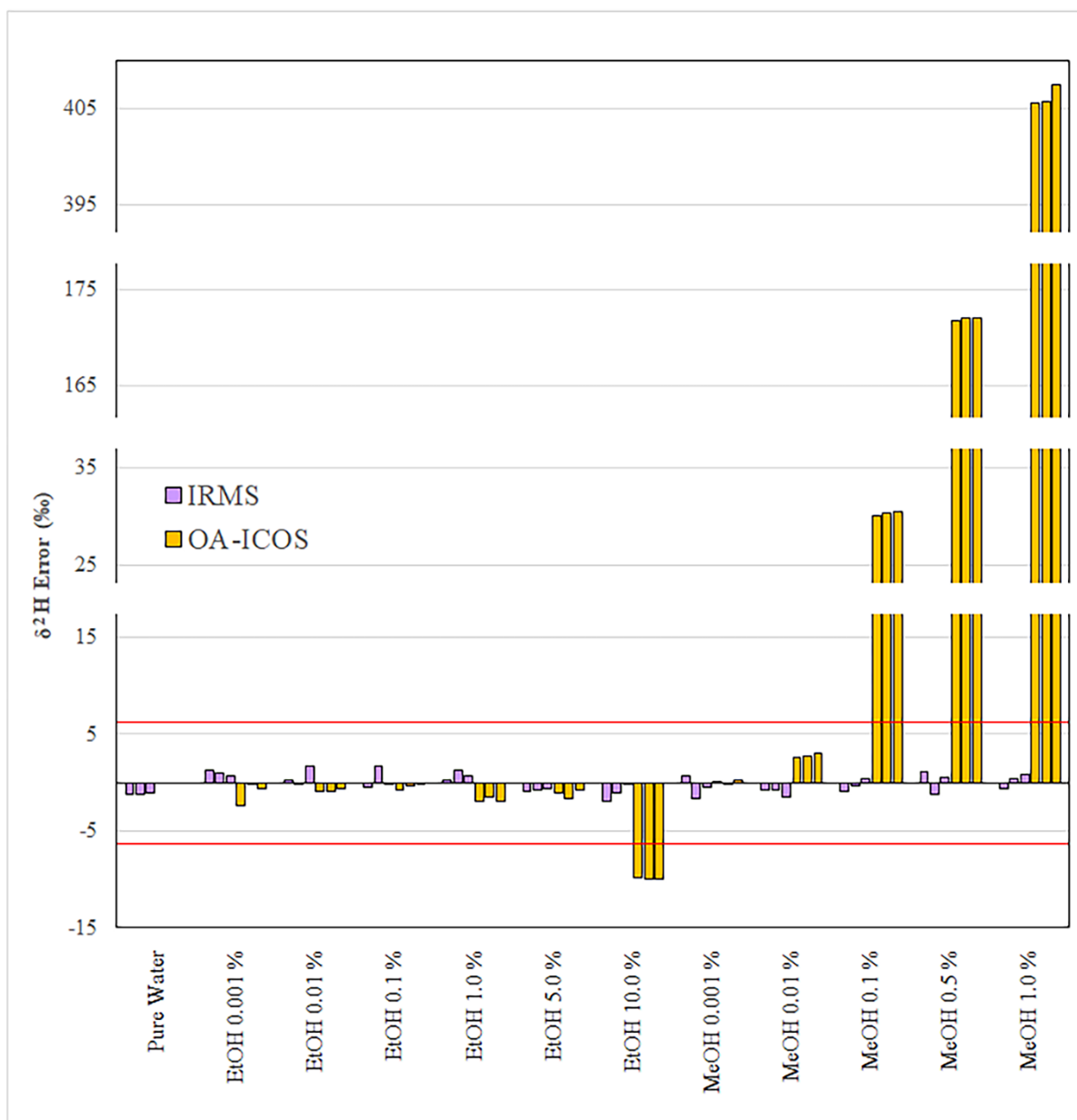
**Figure 4.2.** Dual isotope plots detailing the mean stable isotope results produced by IRMS analysis for the noted level of MeOH and EtOH contamination. Plot A shows the effect of MeOH contamination on IRMS stable isotope results. Plot B shows the effect of EtOH contamination on IRMS results. Data points are the mean (n=3) for each contamination level. Local meteoric water line (LMWL) equation:  $\delta^2\text{H} = 7.7 \times \delta^{18}\text{O} - 1.2\text{‰}$ <sup>[18]</sup>



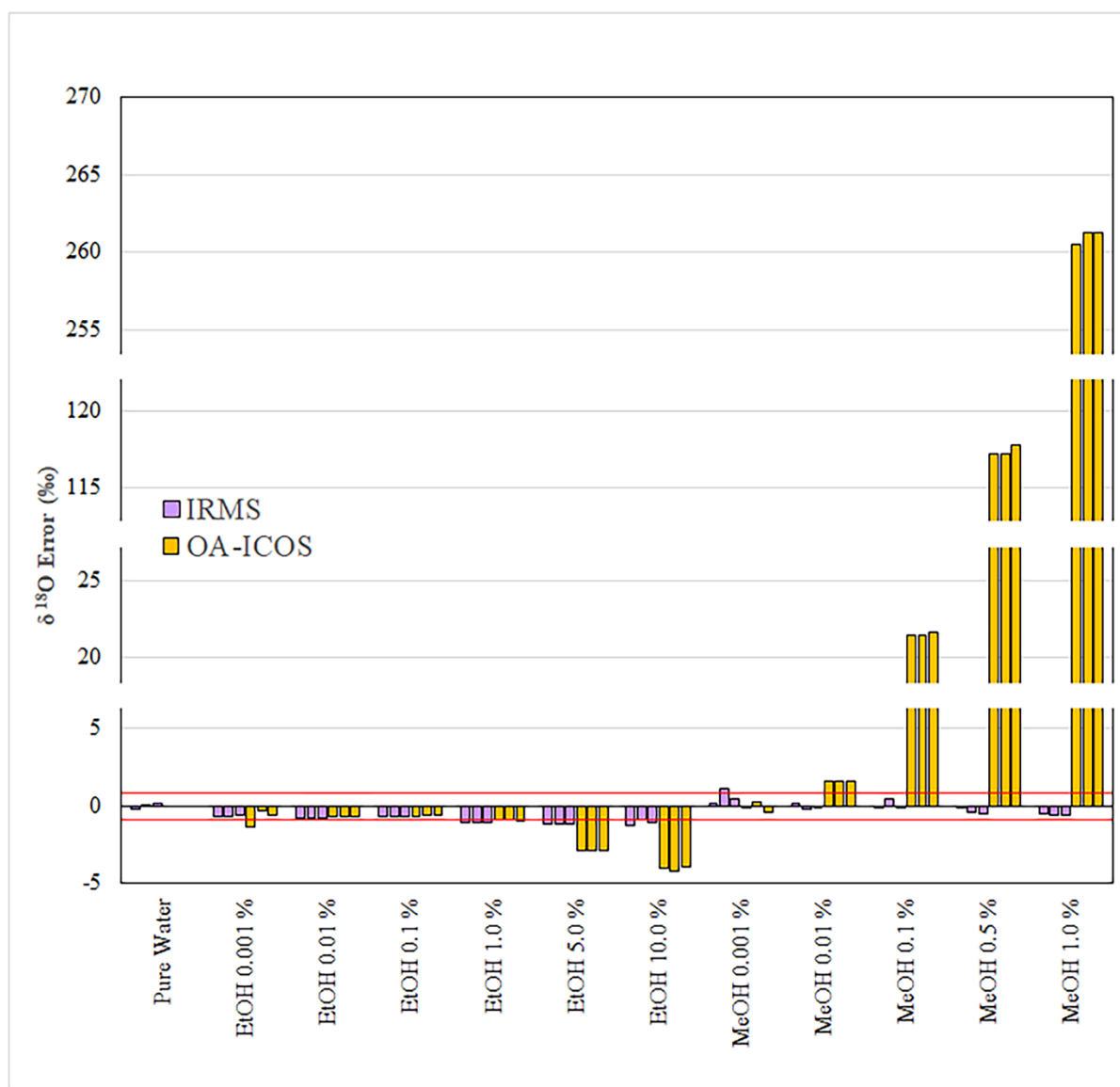
**Figure 4.3.** Dual isotope plots detailing the mean stable isotope results produced by OA-ICOS for liquid analysis at the noted level of MeOH and EtOH contamination. Plot A shows the effect of MeOH contamination on OA-ICOS stable isotope results. Plot B shows the effect of EtOH contamination on OA-ICOS results. Data points are the mean (n=3) for each contamination level. Note that the scales are different for each of the below plots as MeOH and EtOH contamination had varying effects on returned isotopic results. Local meteoric water line (LMWL) equation:  $\delta^2\text{H} = 7.7 \times \delta^{18}\text{O} - 1.2\text{‰}$ <sup>[18]</sup>.



**Figure 4.4.**  $\delta^2\text{H}$  errors (‰) associated with various MeOH and EtOH contamination concentrations in contaminant–water mixtures. The red solid lines represent the accuracy thresholds based on the maximum accepted bias (MAB) established by the International Agency of Atomic Energy ( $\pm 6\text{‰}$  for  $\delta^2\text{H}$ ). The errors were calculated as the differences between the dilutions measured stable isotope values and the DIw control stable isotope values produced by OA-ICOS. Note that the y-axis is not continuous.

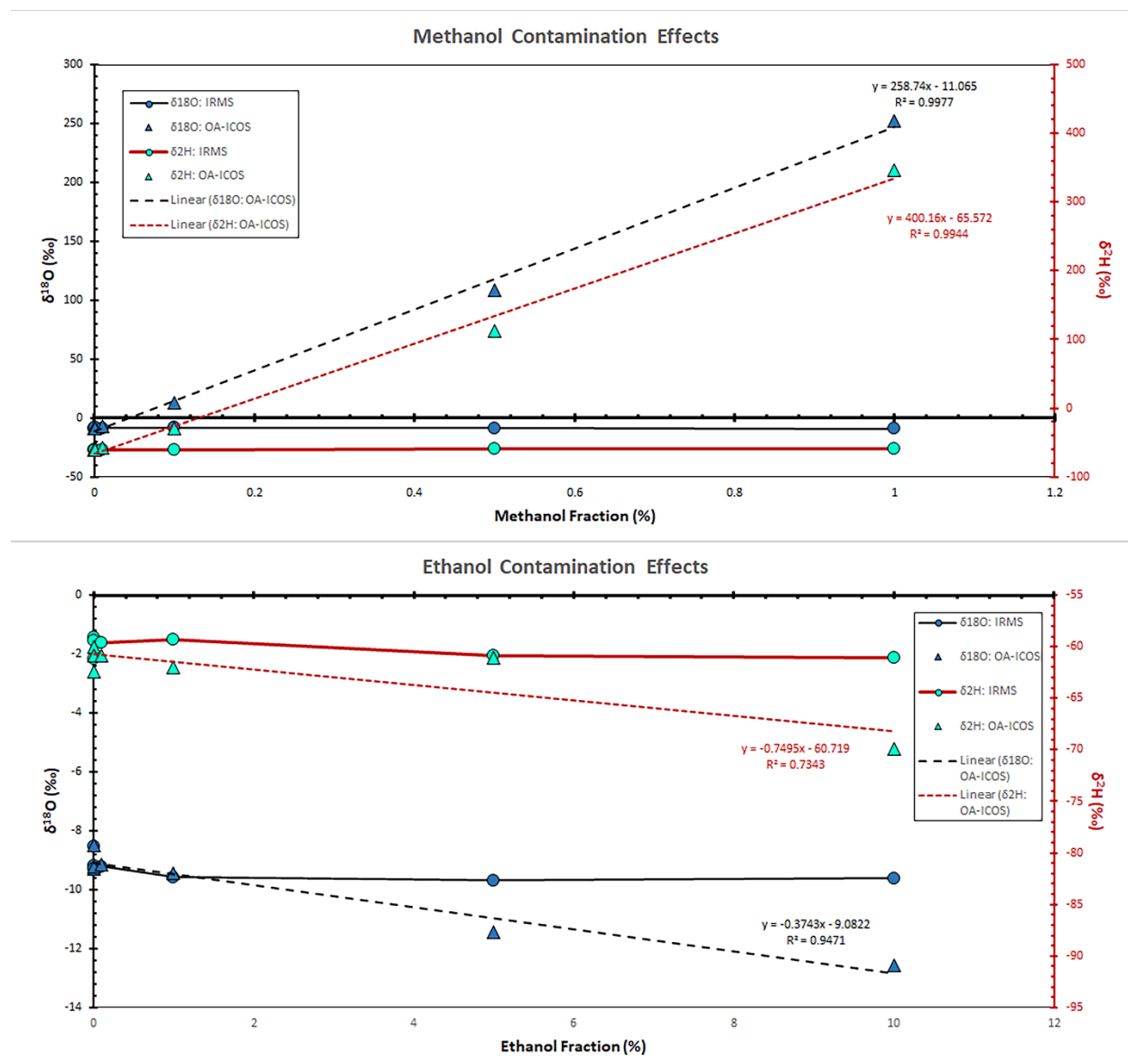


**Figure 4.5.**  $\delta^{18}\text{O}$  errors (‰) associated with various MeOH and EtOH contamination concentrations in contaminant–water mixtures. The red solid lines represent the accuracy thresholds based on the maximum accepted bias (MAB) established by the International Agency of Atomic Energy ( $\pm 0.8\text{‰}$  for  $\delta^{18}\text{O}$ ). The errors were calculated as the differences between the dilutions measured stable isotope values and the DIw control stable isotope values produced by OA-ICOS. Note that the y-axis is not continuous.

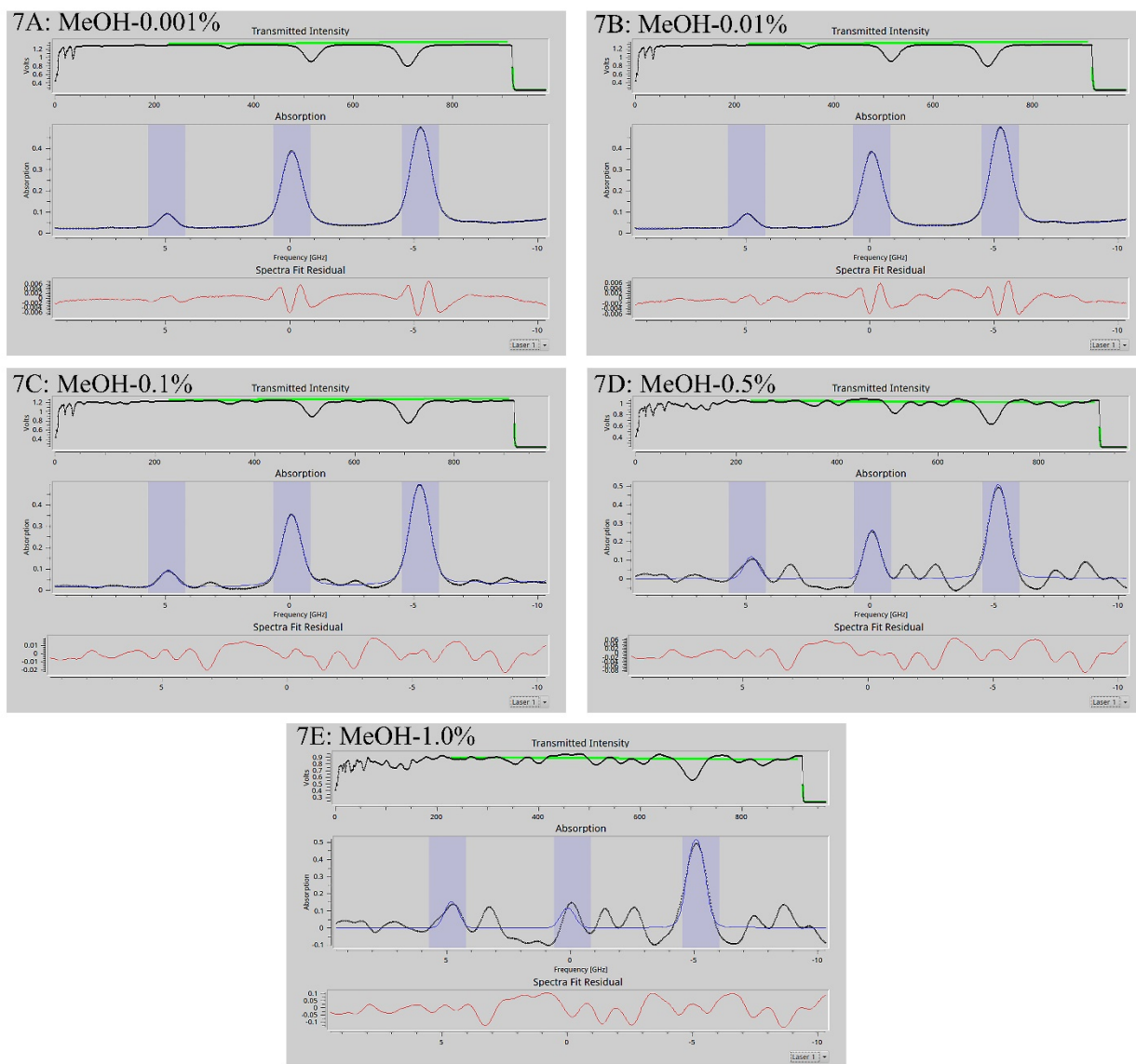




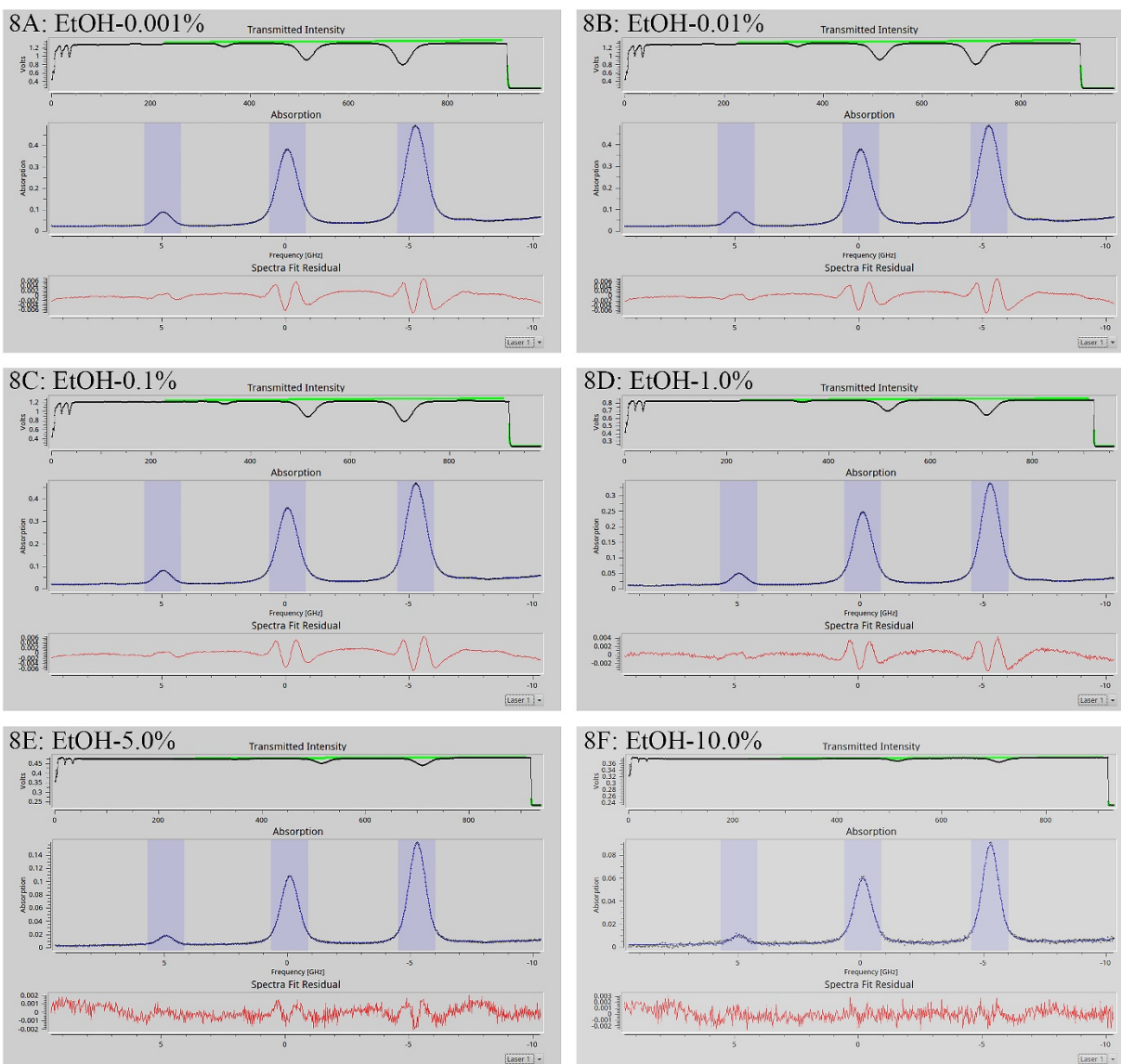
**Figure 4.6.** MeOH/water and EtOH/water mixture results as analysed via IRMS and OA-ICOS. The plots show the offsets of the OA-ICOS results from the IRMS results for each contaminant, at each concentration level. The offsets are linear functions of the MeOH and EtOH fractions, respectively. The dependance of the results on MeOH is far greater than for EtOH.



**Figure 4.7.** Compiled screenshots of output monitor on OA-ICOS showing Absorption (Ab) plots (center graph) and Spectral Fit Residual (SFR) plots (bottom graph) Screenshots taken in real time during DVE analysis via OA-ICOS in vapour-mode, of MeOH/water mixtures at 0.001%, 0.01%, 0.1%, 0.5%, and 1% contaminant concentration. Note in 7B, that we begin to see fluctuations due to MeOH contamination in the SFR plot between normal peaks, but not in the Ab plot. In 7C we begin to see fluctuations in the Ab plot due to MeOH contamination, and the SFR plot shows significant fluctuation. In 7D and 7E both the Ab and SFR plots show significant fluctuations, indicating strong MeOH contamination.



**Figure 4.8.** Compiled screenshots of output monitor on OA-ICOS showing Absorption (Ab) plots (center graph) and Spectral Fit Residual (SFR) plots (bottom graph) Screenshots taken in real time during DVE analysis via OA-ICOS in vapour-mode of EtOH/water mixtures at 0.001%, 0.01%, 0.1%, 1.0%, 5.0%, and 10.0% contaminant concentration. Note in 8D that we begin to see minor noise on the SFR plot due to EtOH contamination, but no fluctuations in the Ab plot. In 8E we begin to see more significant noise in the SFR plot, and barely noticeable indications of noise in the Ab plot. In 8F we see substantial noise in the SFR plots to the extent that the peaks and troughs are drowned out in. 8E shows more noticeable noise in the Ab plot, but it isn't until 8F at EtOH 10.0% that the noise becomes very clear in the Ab plot.



## Chapter 5: Conclusion.

Since the pioneering work of Dawson and Ehleringer<sup>[1]</sup> the stable isotopes of hydrogen and oxygen ( $^2\text{H}/^1\text{H}$  and  $^{18}\text{O}/^{16}\text{O}$ ) have become a widely used tool in the field of ecohydrology. They have been used to investigate plant water sourcing at the laboratory scale<sup>[2, 3]</sup>, all the way up to the ecosystem level<sup>[4, 5]</sup>. They are used to investigate differences in isotopic composition of water within various parts of individual plants<sup>[6-8]</sup>, and are the core evidence underlaying the ecohydrological separation hypothesis (two water world hypothesis)<sup>[9, 10]</sup>. Increasingly, water stable isotopes are being used in agricultural research exploring crop water use strategies, water use efficiency, and irrigation water management strategies<sup>[11, 12]</sup>. However, a critical component of these studies is the ability to extract isotopically unfractionated analytes, representative of the water stream of interest (typically the transpiration stream) from the plant material. A variety of extraction-analysis methods exist to extract analytes from plants for subsequent stable isotope analysis, and until recently it was thought that these methods all returned isotopically similar analytes. But, work by Orlowski *et al.*<sup>[13]</sup> showed that the accuracy and interchangeability of isotopic data provided by various soil water extraction-analysis methods can vary greatly. The accuracy and interchangeability of isotopic data provided by the various extraction-analysis methods is of critical importance, as divergence as little as 10 to 15‰ ( $\delta^2\text{H}$ ) and 2 to 4‰ ( $\delta^{18}\text{O}$ ) are the difference between showing plant use of mobile soil water, or bulk (immobile or matrix) soil water in some studies. My Masters research thus focused on exploring the potential differences between six common lab-based plant analyte extraction systems, and the effects of co-extracted organic compounds on results returned by spectrometric water analysis approaches.

This thesis had two main objectives:

1. Inter-compare the methods of plant water extraction for stable isotope analysis of  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  values, to determine if these extraction systems return accurate, interchangeable results.
2. Examine how the presence of co-extracted organic contaminants like methanol and ethanol in plant water samples affects stable isotope results returned by the LGR OA-

ICOS; and then assess the effectiveness of the Absorption plot and the Spectra Fit Residuals plot at detecting organic contamination during vapour-mode analysis via OA-ICOS.

The driving impetus for this research were calls by Sprenger *et al.* <sup>[14]</sup> and McDonnell <sup>[10]</sup> for intercomparison of the various soil and plant water extraction methods. A further drive was the findings of Orlowski *et al.* <sup>[13]</sup>, showing that five commonly used soil water extraction methods produced significantly different results. This work implied that methodological effects may also impact the isotopic results of analytes extracted by the various plant analyte extraction-analysis methods. I addressed this call for intercomparison in Chapter 2 and Chapter 3. Additionally, previous work had shown that methanol (MeOH) and ethanol (EtOH) contamination affected isotopic results returned by the Picarro cavity ring down spectroscopy (CRDS) system <sup>[15, 16]</sup>. West *et al.* <sup>[17]</sup> tested the effects of plant extracted analytes, known to have issues with co-extracted organic content on the Los Gatos Research (LGR) off-axis integrated cavity output spectroscopy (OA-ICOS) system and the CRDS system. They showed that each system produced significantly different results when exposed to organic contaminated plant water. However, research had not directly quantified the effects of known concentrations of MeOH and EtOH contamination on the OA-ICOS system. In Chapter 4 this research gap was addressed.

## 5.1 Findings of Chapter 2

In Chapter 2 <sup>[18]</sup> we performed an inter-method comparison of six common plant water extraction techniques: direct vapour equilibration (DVE), microwave extraction coupled with on-line OA-ICOS analysis (ME-OA-ICOS), two unique versions of cryogenic vacuum distillation (CVD-1 and CVD-2), centrifugation, and high pressure mechanical squeezing (HPMS). These methods were applied to four isotopically unique plant portions (head, stem, leaf, and root crown) of spring wheat (*Triticum aestivum* L.). When possible, we analysed extracted plant water via spectrometric (OA-ICOS) and mass based (IRMS) analysis systems to account for analytical differences between the two approaches and potential impacts of co-extracted organic contaminants. We also quantified the MeOH and EtOH content of water extracted by the methods that produced a liquid analyte.

Similar to the findings of Orlowski *et al.* <sup>[13]</sup> we discovered that the tested extraction-analysis methods produced significantly different results ( $p \leq 0.05$ ) depending upon the plant portion that analytes were extracted from. However, we strongly caution readers when attempting to compare the result of our study with the Orlowski *et al.* <sup>[13]</sup> intercomparison, as the water carrying media (soil vs plant matter) are highly different from one another with regard to relative inertness, internal chemistry, effects on water isotope ratios and presence of co-extractable compounds <sup>[18]</sup>. Additionally, we found that the tested extraction-analysis methods co-extracted varying amounts of MeOH and EtOH, and that this MeOH and EtOH content can result in significant differences between results returned by OA-ICOS and IRMS analysis approaches. Specifically, the CVD-1 system, that extracted plant water at 100 °C, showed little difference between OA-ICOS and IRMS analysis results even though MeOH and EtOH content caused the spectral contamination detection software (LGR LWIA-SCI) to flag all samples for contamination. The CVD-2 system, using 200 °C as an extraction temperature, produced an order of magnitude greater MeOH and EtOH content in the extracted water than that produced by CVD-1, and consequently there were significant differences between OA-ICOS and IRMS results.

Assessing the accuracy of results produced in an intercomparison of this type (using plant extracted analytes) is difficult, as defining a reference value used in z-score analysis is not possible. As such we approach the question of accuracy in terms of analyte purity and methodological effects on extracted water. Since we are interested in answering question about plant water sourcing, and therefore the isotopic composition of the transpiration water, any extraction-analysis approach that also co-extracted substantial amounts of organics has the potential to be less accurate, as these co-extracted compounds can influence the final isotopic results returned by both OA-ICOS and to a lesser extent IRMS analysis approaches. From this line of reasoning we suggest that the DVE system may be the most appropriate system for investigating the isotopic composition of the transpiration stream in plant samples, as this approach operates at room temperature and may minimize the amount co-extracted organic compounds present in the analysis stream. Furthermore, we suggest that each extraction-analysis approach will induce methodological effects related to which specific water, and H & O pools

within the plant that are extracted. Therefore, depending on the plant portion of interest and method used, a unique set of organic compounds and potential water pools within the plant unrelated to water transport and whose isotopic composition may be dominated by solute content, can be extracted. This will thus impact the final returned isotopic results.

## 5.2 Findings of Chapter 3

In Chapter 3 we further investigated potential differences in stable isotope results produced by the DVE and CVD-2 extraction-analysis approaches. In this study we applied the DVE and CVD-2 extraction-analysis systems to a larger number of spring wheat samples collected over an eight-week study period. CVD-2 extracted analytes were analysed via IRMS while DVE analysis took place on an OA-ICOS system. The driving impetus of this study was that in the findings of Chapter 2 we did not see significant differences between the stable isotope results produced by the DVE and CVD-2 systems. The lack of significant differences in results was contrary to our main reasoning for why we were seeing differences between some of the various extraction-analysis systems results in Millar *et al.* <sup>[18]</sup>. We sought to improve our understanding of differences between these systems by doing further study with a higher number of samples for comparison. As we expected, for all eight weeks, except in week 7 where irrigation occurred shortly before sampling,  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  values of analytes produced by DVE and CVD-2 were significantly different.

## 5.3 Chapter 2 and 3 Implications and Limitations

The findings of Chapter 2 <sup>[18]</sup> and Chapter 3 have important implications for any research fields using isotopic results from plant extracted analytes. This work suggests that the various extraction-analysis systems should not be used interchangeably and that researchers should choose an extraction approach whose methodological effects on the pools of water accessed within the plant sample are appropriate to their specific research questions. Furthermore, the CVD-1 and CVD-2 results from Chapter 2 were more depleted than all other approaches and since CVD is the most widely used extraction-analysis method in the literature <sup>[13]</sup>, this will have implication for conclusions drawn by studies using this approach. There are also specific limitations to the findings of Chapter 2 <sup>[18]</sup>. Specifically, these findings should only be extended

to other herbaceous species, as woody plant tissue may have greater issues related to co-extracted organic compounds. Additionally, we were not able to quantify and identify the volumes and types of other co-extracted organic compounds like proteins, carbohydrates, terpenes etc., some of which may also impact stable isotope results. We also note that the low number of samples used in our intercomparison limited out statistical power and that future method intercomparisons should seek to use a higher number of samples. In Chapter 3 we addressed this specific issue by collecting a larger number of samples for analysis on DVE and CVD-2 approaches.

#### 5.4 Findings of Chapter 4

In Chapter 4 we created a series of methanol (MeOH) and ethanol (EtOH) contaminated water samples and analysed them for their stable isotope composition in liquid and vapour-mode via the OA-ICOS approach and in liquid-mode via IRMS. We tested the Absorption plot and Spectra Fit Residuals plot, components of the OA-ICOS system, for their ability to detect organic contamination in water samples during vapour mode analysis. Similar to previous work <sup>[15-17]</sup>, we found that MeOH and EtOH contamination does indeed affect isotopic results returned by isotope ratio infrared spectroscopy (IRIS) approaches. For both  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  ratios, OA-ICOS results showed substantial deviations from IRMS and reference water results for methanol-water mixtures at greater than 0.01% concentrations. In fact, MeOH-water mixtures showed deviations as large as 406‰ ( $\delta^2\text{H}$ ) and 261‰ ( $\delta^{18}\text{O}$ ) relative to the reference water isotopic composition when analysed in liquid-mode via OA-ICOS. EtOH-water mixtures only showed noticeable deviations between OA-ICOS and reference water results at 5% and 10% concentrations. Deviations as large as -9.8‰ ( $\delta^2\text{H}$ ) and -4.1‰ ( $\delta^{18}\text{O}$ ) relative to the control water isotopic composition were measured during liquid analysis of EtOH-water mixtures on OA-ICOS. Interestingly, in liquid-mode analysis via OA-ICOS, it was the  $\delta^2\text{H}$  results that were most strongly affected by the presence of contaminants, whereas in vapour-mode, the  $\delta^{18}\text{O}$  results were the far more strongly affected of the two isotopic ratios. Unlike previous findings which showed that organic contamination caused CRDS results to skew negatively and OA-ICOS results to skew positively in the presence of organic contaminants <sup>[17]</sup>, we show that MeOH causes OA-ICOS results to skew positively, while EtOH causes OA-ICOS result to skew



negatively. When also found that the Spectra Fit Residuals (SFR) plot is a useful tool for detecting organic contamination of samples analysed in vapour-mode via OA-ICOS. The SFR plot detected MeOH contamination at concentrations  $\geq 0.01\%$ , and EtOH contamination at concentrations  $\geq 0.1\%$  in vapour-mode on OA-ICOS. Both noted concentrations (0.01% MeOH and 0.1% EtOH) are when isotopic results produced by the OA-ICOS began to diverge from IRMS results. This is a highly useful finding as contaminant detection software for use during vapour-mode analysis does not yet exist. Most usefully, since the output of the SFR plot is based on raw data within the OA-ICOS system, it may be possible to develop a correction approach for contaminant affected results produced during vapour-mode analysis, though these corrections will likely be machine specific as opposed to applicable to all analysis machines produced by the same manufacturer.

### **5.5 Chapter 4 Implications and Limitations**

While the findings of Chapter 4 are not entirely unique, they serve to confirm the well known problems related to organic compound contamination in plant extracted water and their effect on results returned by IRIS analysis approaches. As IRIS approaches are cheaper and faster to run than their IRMS counterpart, they have been increasingly utilized for soil water analysis. Soil water typically does not suffer from organic contamination on the levels that plant extracted analytes do. Due to the speed, cost effectiveness and portability of the OA-ICOS approach, researchers are now also attempting to use this method of analysis with plant analytes. The findings of Chapter 4 further cement the need for pre-processing filtration and/or post processing correction techniques like those detailed in Schultz *et al.* <sup>[19]</sup> and Martin-Gomez *et al.* <sup>[16]</sup> if the IRIS analysis approach is to be used on plant extracted analytes. Until issues related to co-extracted organic compound caused errors in stable isotope results can be addressed for IRIS analysis approaches, we urge caution in their use in analyzing plant extracted analytes. The stable isotope composition of the MeOH and EtOH used in this study will have likely influenced the returned isotopic results, in addition to the spectral interference issues related to MeOH and EtOH O-H bonds. Furthermore, since we utilized 95% EtOH and 98% MeOH as the contaminant source, the isotopic composition of the water in these non-pure contaminants would

have also impacted analytical results. Additionally, a greater number of samples could have been used in the vapour-mode analyses to improve our statistical power.

### **5.6 Future Recommendations**

Future research should seek to build on the findings of this thesis work. Specifically inter method comparisons of available plant analyte extraction techniques should be carried out on a variety of woody plant species to better elucidate methodological effects on returned plant analytes, as well as expanding upon our understanding of problems related to increased co-extracted organic content related to these woody species. It may be that due to the smaller volume of water held in herbaceous species relative to trees, that co-extracted organic compounds could be more or less problematic depending on their volumes relative to the water volume of the plant. However, little work has been done to quantify relative volumes, types, and isotopic composition of organic compounds co-extracted with plant water from various species of interest. Indeed, seasonal, and daily water cycling patterns within plants may also impact organic compound content in various plant portions, which in turn will impact results based on time of sampling. This should therefore be studied and considered during future research and sampling planning. The finding that different methods of extraction may be accessing different water, organic compounds, and atomic H and O pools within the plant also implies a need for better understanding of the movement, potential fractionation zones, and cycling of water within the plant. Compartmental modelling of plant water and organic compound pools utilizing real world bulk plant water (CVD extracted), and live plant transpiration and internal water isotopic composition data (collected by in-situ and DVE approaches) may be a powerful approach for such understanding. The findings of my thesis work has important implications for the tools used to access and analyse plant extracted analytes in the fields of ecohydrology, agricultural water use and management, and global hydrological modelling.

## 5.7 References

- [1] Dawson T E, Ehleringer J R. Stream side trees that do not use stream water. *Nature* 1991; 350(335-337).
- [2] Bakhshandeh S, Kertesz M A, Corneo P E, Dijkstra F A. Dual-labeling with  $^{15}\text{N}$  and  $\text{H}_2^{18}\text{O}$  to investigate water and  $\text{N}$  uptake of wheat under different water regimes. *Plant and Soil* 2016; 408(1-2):429-441.
- [3] Barnard R L, de Bello F, Gilgen A K, Buchmann N. The  $\text{d}^{18}\text{O}$  of root crown water best reflects source water  $\text{d}^{18}\text{O}$  in different types of herbaceous species. *Rapid Commun Mass Spectrom* 2006; 20(24):3799-3802.
- [4] Evaristo J, McDonnell J J, Scholl M A, Bruijnzeel L A, Chun K P. Insights into plant water uptake from xylem-water isotope measurements in two tropical catchments with contrasting moisture conditions. *Hydrological Processes* 2016; 30(18):3210-3227.
- [5] Good S P, Noone D, Kurita N, Benetti M, Bowen G J.  $\text{D}_2$  isotope ratios in the global hydrologic cycle. *Geophysical Research Letters* 2015b; 42(12):5042-5050.
- [6] Zhao L, Wang L, Cernusak L A, Liu X, Xiao H, Zhou M, Zhang S. Significant difference in hydrogen isotope composition between xylem and tissue water in *Populus euphratica*. *Plant Cell Environ* 2016a; 39(8):1848-1857.
- [7] Cernusak L A, Wong S C, Farquhar G D. Oxygen isotope composition of phloem sap in relation to leaf water in *Ricinus communis*. *Functional Plant Biology* 2003; 30(10):1059.
- [8] Cernusak L A, Barbour M M, Arndt S K, Cheesman A W, English N B, Feild T S, Helliker B R, Holloway-Phillips M M, Holtum J A, Kahmen A, McInerney F A, Munksgaard N C, Simonin K A, Song X, Stuart-Williams H, West J B, Farquhar G D. Stable isotopes in leaf water of terrestrial plants. *Plant Cell Environ* 2016; 39(5):1087-1102.
- [9] Brooks J R, Barnard H R, Coulombe R, McDonnell J J. Ecohydrologic separation of water between trees and streams in a mediterranean climate. *Nature Geoscience* 2010; 3(2):100-104.
- [10] McDonnell J J. The two water worlds hypothesis ecohydrologic separation of water between trees and streams. *WIREs: Water* 2014; 1(4):323-329.
- [11] Wang P, Song X, Han D, Zhang Y, Zhang B. Determination of evaporation, transpiration and deep percolation of summer corn and winter wheat after irrigation. *Agricultural Water Management* 2012; 105(32-37).
- [12] Zhao P, Tang X, Zhao P, Tang J. Dynamics of water uptake by maize on sloping farmland in a shallow entisol in southwest china. *Catena* 2016b; 147(511-521).
- [13] Orłowski N, Pratt D L, McDonnell J J. Intercomparison of soil pore water extraction methods for stable isotope analysis. *Hydrol Process* 2016b; 30(19):3434-3449.
- [14] Sprenger M, Herbstritt B, Weiler M. Established methods and new opportunities for pore water stable isotope analysis. *Hydrol Process* 2015; 29(25):5174-5192.
- [15] Brand W A, Geilmann H, Crosson E R, Rella C W. Cavity ring-down spectroscopy versus high-temperature conversion isotope ratio mass spectrometry; a case study on  $\delta(2)\text{H}$  and  $\delta(18)\text{O}$  of pure water samples and alcohol/water mixtures. *Rapid Commun Mass Sp* 2009; 23(12):1879-1884.
- [16] Martin-Gomez P, Barbeta A, Voltas J, Penuelas J, Dennis K, Palacio S, Dawson T E, Ferrio J P. Isotope-ratio infrared spectroscopy: A reliable tool for the investigation of plant-water sources? *New Phytol* 2015; 207(3):914-927.

- [17] West A G, Goldsmith G R, Brooks P D, Dawson T E. Discrepancies between isotope ratio infrared spectroscopy and isotope ratio mass spectrometry for the stable isotope analysis of plant and soil waters. *Rapid Commun Mass Sp* 2010; 24(14):1948-1954.
- [18] Millar C, Pratt D, Schneider D J, McDonnell J J. A comparison of extraction systems for plant water stable isotope analysis. *Rapid Commun Mass Sp* 2018; 32(13):1031-1044.
- [19] Schultz N M, Griffis T J, Lee X, Baker J M. Identification and correction of spectral contamination in 2h/1h and 18o/16o measured in leaf, stem, and soil water. *Rapid Commun Mass Spectrom* 2011; 25(21):3360-3368.